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TITLE: Lung Squamous Cell Carcinoma Stem Cells as Immunotherapy Targets

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14. ABSTRACT Lung squamous cell carcinoma (SCC) is the second most common type of lung cancer, and immunotherapy is a promising new treatment for SCC. In the <i>Lkb1/Pten</i> mouse model of SCC, the cancer stem cells (CSCs) have high expression of the immune evasion molecule PD-L1, a ligand for the PD1 molecule expressed on T-cells known to mediate T-cell suppression. Antibody therapy that effectively inhibits PD1/PD-L1 interactions has had exciting success in SCC patients, yet the specific cellular mechanisms of therapy efficacy or paths to resistance are unknown. <i>Lkb1/Pten</i> mice with established SCC detected by MRI were treated with anti-PD1 antibody. There was no significant difference in the rate the tumor growth between the two groups, indicating that this mouse model may be a good model of immunotherapy resistance. CSCs were isolated by FACS, quantified, and reserved for future RNA-Seq studies. Interestingly, mice that received PD-L1 treatment showed an increased number of CSCs. Gene expression profiling will be used to learn what pathways govern SCC CSCs. These studies will yield cogent design and deployment of clinical trials with the best rational combination of agents for these acquired resistant and non-responsive lung cancer patients.					
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Table of Contents

	<u>Page</u>
1. Introduction.....	4
2. Keywords.....	4
3. Accomplishments.....	4
4. Impact.....	12
5. Changes/Problems.....	13
6. Products.....	13
7. Participants & Other Collaborating Organizations.....	13
8. Special Reporting Requirements.....	None
9. Appendices.....	None

1. INTRODUCTION

Lung squamous cell carcinoma (SCC) is the second most common type of lung cancer, and immunotherapy is a promising new treatment for SCC. In the *Lkb1/Pten* mouse model of SCC, the cancer stem cells (CSCs) have high expression of the immune evasion molecule PD-L1, a ligand for the PD1 molecule expressed on T-cells known to mediate T-cell suppression. Antibody therapy that effectively inhibits PD1/PD-L1 interactions has had exciting success in SCC patients, yet the specific cellular mechanisms of therapy efficacy or paths to resistance are unknown. *Lkb1/Pten* mice with established SCC detected by MRI were treated with anti-PD1 antibody. There was no significant difference in the rate the tumor growth between the two groups, indicating that this mouse model may be a good model of immunotherapy resistance. CSCs were isolated by FACS, quantified, and reserved for future RNA-Seq studies. Interestingly, mice that received PD-L1 treatment, yet did not have any significant change in tumor burden, showed an increased number of CSCs. These results we obtained with this round of experiments was quite different than what we observed in our first round of studies in which *Lkb1/Pten* mice had reduced tumor burden with anti-PD1 treatment. We also tested the effect of the mTor inhibitor Torin2 on tumor burden alone and in combination with immunotherapy; our initial studies suggest that dual therapy with mTor inhibitor and immunotherapy is superior to immunotherapy alone. Critical to our hypothesis that cancer stem cells may be a target of immunotherapy, in our first round of studies in which dual treatment with mTor inhibitor and immunotherapy was beneficial, the abundance of Sca1+ NGFR+ cells (the CSCs) was reduced in treated mice. Our data from multiple immunotherapy treatment studies to date suggest that resistance or lack of response to immunotherapy may be due to failure to effectively target cancer stem cells. Our next steps will be to perform gene expression studies to determine how cancer stem cells change when immunotherapy is not successful. Gene expression profiling will be used to learn what pathways govern SCC CSCs. These studies will yield cogent design and deployment of clinical trials with the best rational combination of agents for these acquired resistant and non-responsive lung cancer patients.

2. KEYWORDS

squamous cell carcinoma (SCC)
cancer stem cells (CSCs)
PD-L1
PD1
Immunotherapy
T cells
immune cells

3. ACCOMPLISHMENTS

What were the major goals of the project?

We hypothesize that SCCs retain a distinct population of cancer stem cells (CSCs) with unique tumor immune-evasion properties, which impact therapeutic response and acquired resistance to both cytotoxic therapies and immunotherapies.

The Specific Aims of this work were to:

- Aim 1. To identify and characterize CSCs in human and murine lung squamous cell carcinoma
- Aim 2. To determine if lung SCC CSCs are specifically targeted by PD1/PD-L1 blockade immunotherapy.
- Aim 3. To determine the mechanisms of acquired or intrinsic resistance to PD1/PD-L1 blockade in murine and human lung SCC, and therapeutic strategies to overcome resistance.

These Aims were to be accomplished by the following Major Tasks:

Major Task 1 To identify and characterize cancer stem cells (CSCs) in human and murine lung squamous cell carcinoma; Major Task 2 To determine if lung squamous cell carcinoma CSCs are specifically targeted

by PD1/PD-L1 blockade immunotherapy; Major Task 3 To determine the mechanisms of acquired or intrinsic resistance to PD1/PD-L1 blockade in murine and human lung SCC, and therapeutic strategies to overcome resistance.

What was accomplished under these goals?

Lung SCC is the second most common type of lung cancer, and immunotherapy is a promising new treatment for this subtype of lung cancer. We created a unique genetically engineered lung SCC model in the mouse driven by alterations of the same pathways found to be impacted in human lung cancers. In this mouse model, initiated by biallelic inactivation of the tumor suppressor genes *Lkb1* (*Stk11*) and *Pten*, tumor cells with the surface markers Sca1 (*Ly6a*) and Nerve Growth Factor Receptor (NGFR) formed tumors when serially transplanted. Thus, the Sca1+/NGFR+ cells represent the tumor-propagating cells, often referred to as cancer stem cells (CSCs), for this model of squamous lung cancer. Characterization of these CSCs revealed high expression of the immune evasion molecule PD-L1, which is a ligand for the PD1 molecule expressed on T-cells known to mediate T-cell suppression. Antibody therapy that effectively inhibits PD1/PD-L1 interactions has had exciting success in SCC patients, yet the specific cellular mechanisms of therapy efficacy or paths to resistance are unknown.

Lkb1/Pten mice with established SCC detected by MRI were treated with anti-PD1 antibody to determine if tumor growth is influenced. The effect of therapy on immune microenvironment and CSC activity was characterized. A total of 15 mice were treated either with PD-1 antibody (7 mice) or with the IgG isotope control (8 mice) in two independent batches (Figure 1 and 2). Treatment started when the tumor burden was between 150 mm³ and 400 mm³ and the treatment duration was 4-5 weeks. MRI was performed after two weeks of treatment and every week after that. There was no significant difference in the rate the tumor growth between the two groups, indicating that this mouse model may be a good model of immunotherapy resistance.

To perform immunoprofiling, 2 mice of the first batch and 3 mice of the second batch were randomly chosen. There was no difference in the immune cell composition of the two mice of the first batch (Figure 5), which matches with the observation that their tumor growth rate was similar too (Figure 3). In contrast, there was a difference in the composition of the immune cells in the second batch (2 mice per treatment group) (Figure 6), which matches with the observation that the two mice that received the PD-1 antibody treatment had a decreased tumor growth rate. The treated mice had fewer tumor associated neutrophils (TANs), more B-cells, CD4 T-cells, and CD8 T-cells.

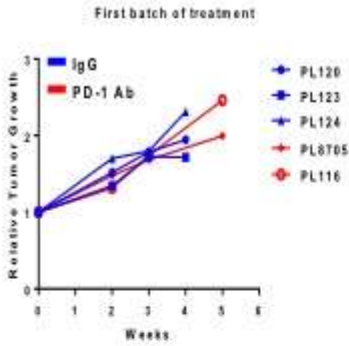


Figure 1

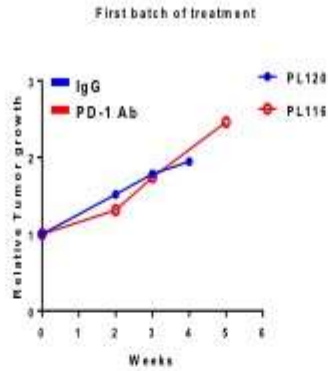


Figure 3

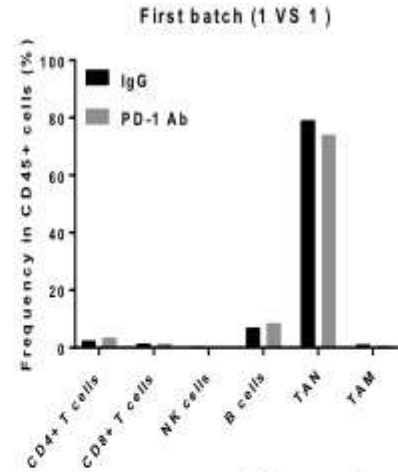


Figure 5

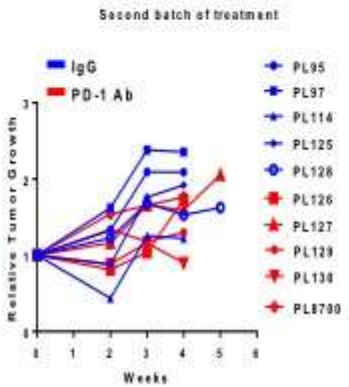


Figure 2

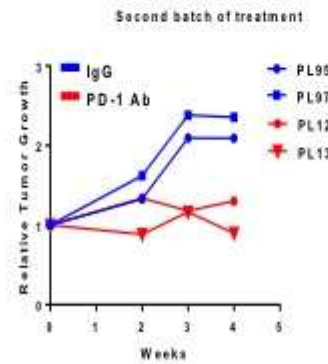


Figure 4

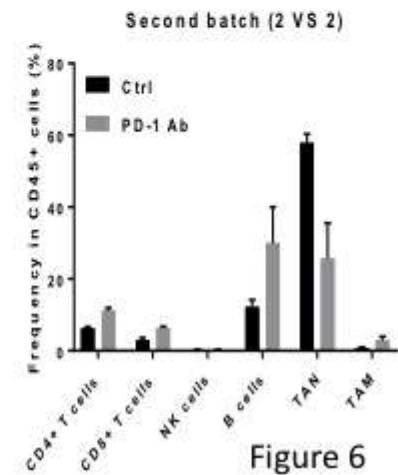


Figure 6

Tumor CSCs were isolated from lung SCC by FACS, quantified, and reserved for future RNA preparation for RNA-Seq gene expression studies. A total of seven mice was used to perform FACS sorting for epithelial tumor cells (EpCAM+), excluding dead cells, CD45+ blood cells and CD31+ endothelial cells. The EpCAM+ cell fraction was divided into four populations depending on expression levels of the surface markers Sca1 and NGFR. Interestingly, the group that received PD-L1 treatment showed an increased number of Sca1+/NGFR+ positive cells and a decreased number of Sca1+/NGFR- cells (Figure 7). Three of the four cell populations (excluding the Sca1-/NGFR+ population because of low cell numbers) were sorted and RNA was collected to perform RNASeq in future. Gene expression profiling will be used to learn what pathways govern SCC CSCs.

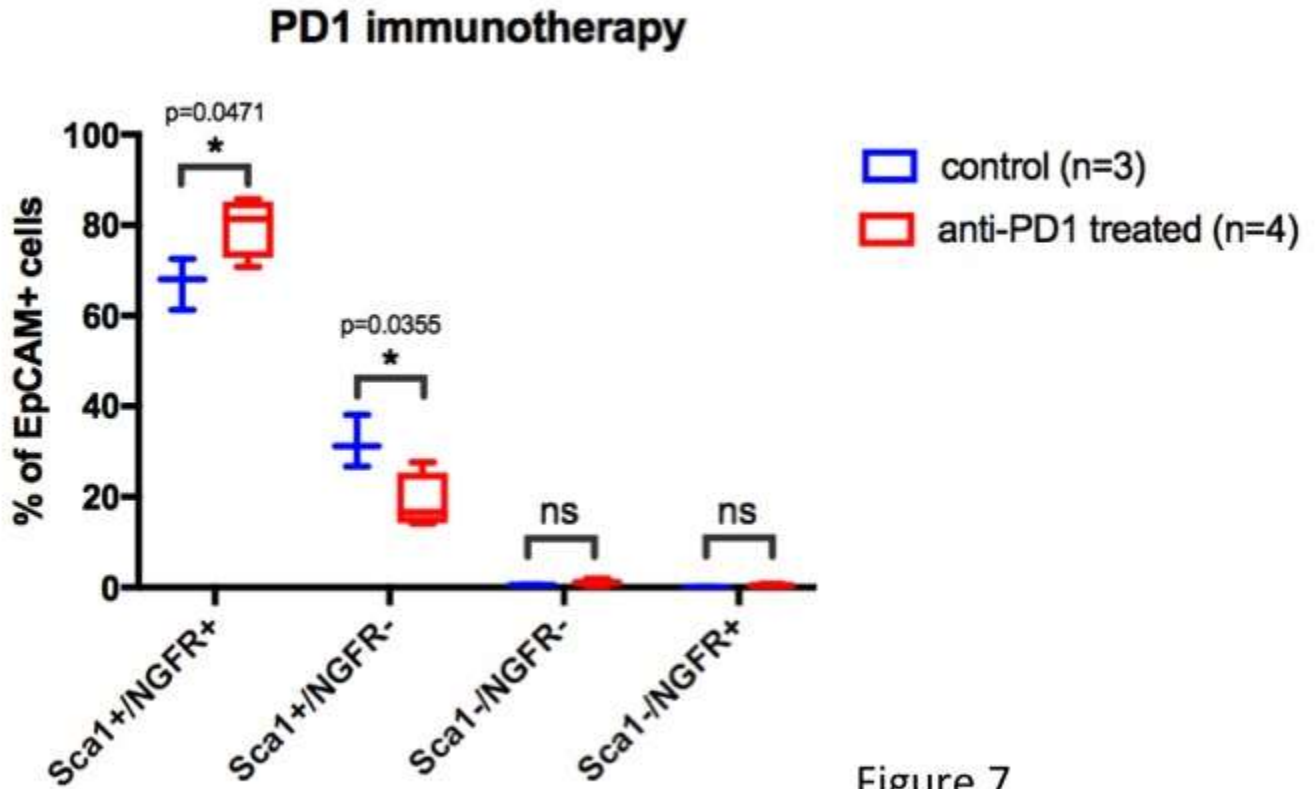


Figure 7

Importantly, the results we obtained with this round of experiments was quite different than what we observed in our first round of studies in which *Lkb1/Pten* mice had reduced tumor burden with anti-PD1 treatment. First, in our pilot study, tumor burden was reduced in mice that received anti-PD1 compared to taxotere-treated mice or untreated controls (Figure 8a). The overall survival of mice that received anti-PD1 was also better than mice that received docetaxol (Figure 8b). Flow cytometry confirmed that the anti-PD1 antibody was hitting its target in these studies (Figure 8c). Thus, when anti-PD1 is effectively targeting PD1, *Lkb1/Pten* mice have beneficial outcomes.

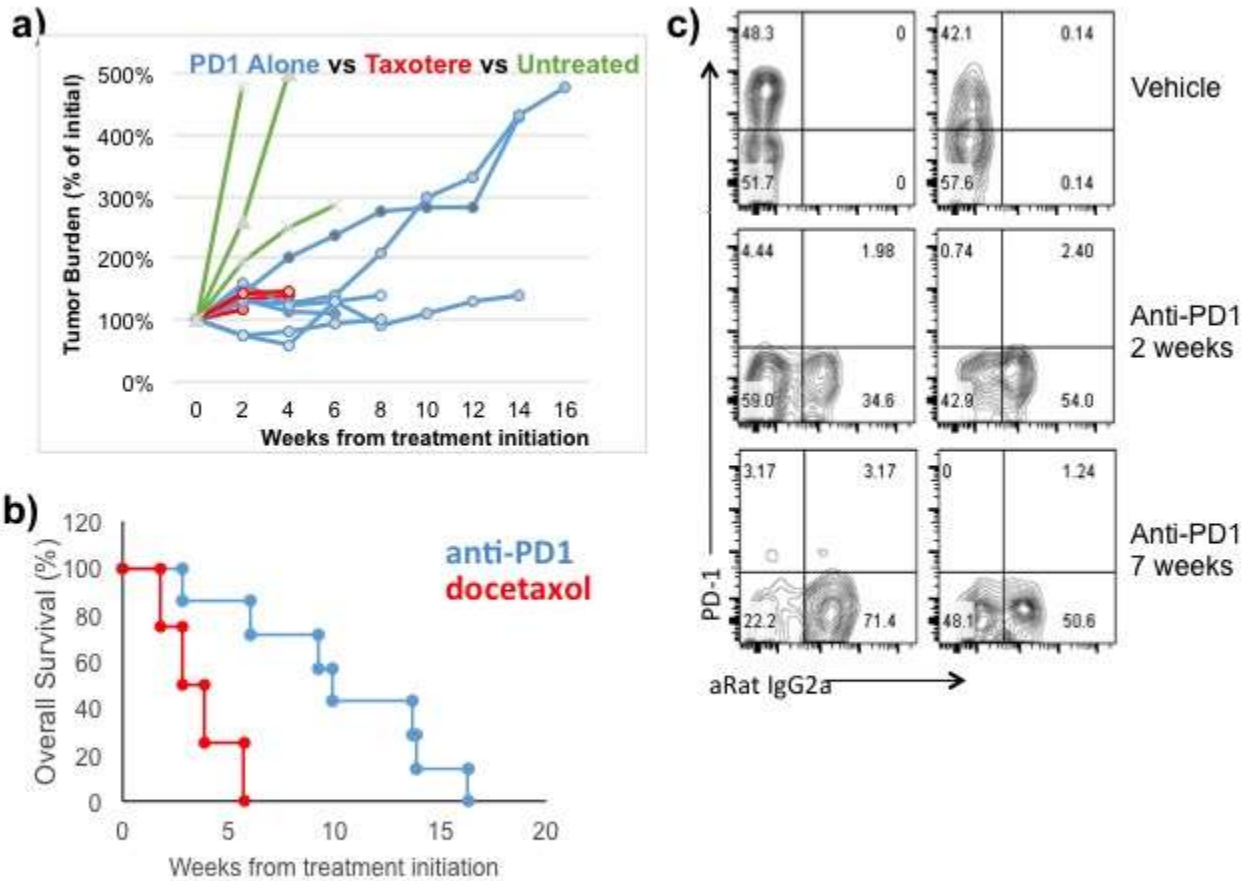


Figure 8: Anti-PD1 immunotherapy significantly increases survival of squamous-tumor bearing mice compared to docetaxol

We also tested the effect of the mTor inhibitor Torin2 on tumor burden alone and in combination with immunotherapy; our initial studies suggest that dual therapy with mTor inhibitor and immunotherapy is superior to immunotherapy alone (Figure 9).

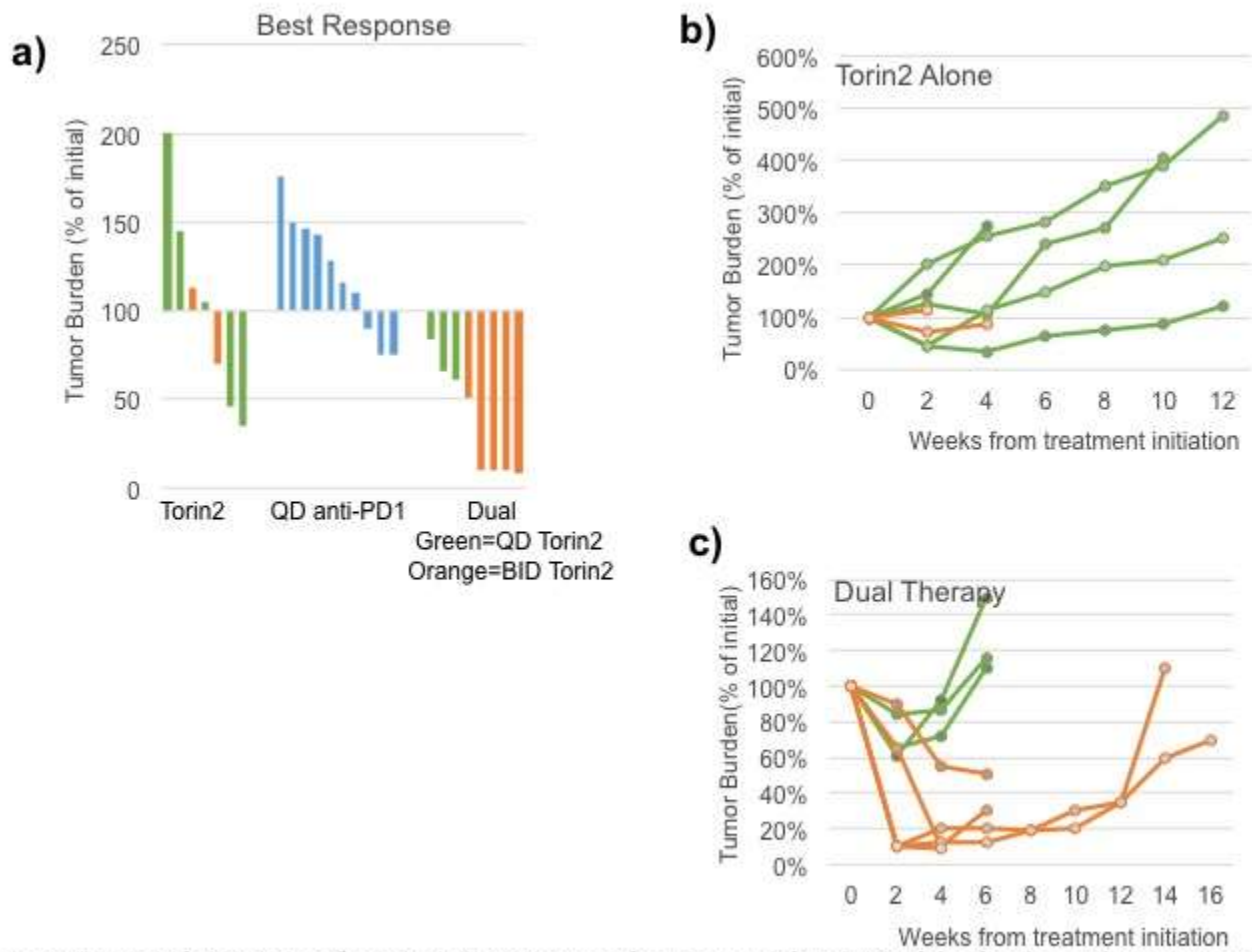


Figure 9: Combination of anti-PD1 immunotherapy with mTOR blockade reduces tumor burden

Critical to our hypothesis that cancer stem cells may be a target of immunotherapy, in our first round of studies in which dual treatment with mTOR inhibitor and immunotherapy was beneficial, the abundance of Sca1+ NGFR+ cells (the CSCs) was reduced in treated mice (Figure 10).

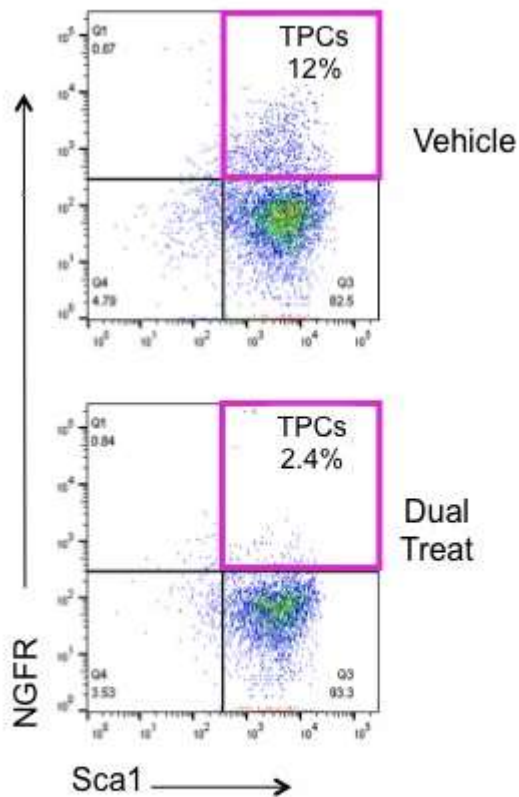


Figure 10: Combination of anti-PD1 immunotherapy with mTOR blockade changes CSC profile

These studies are beginning to uncover mechanisms of tumor maintenance, therapy response, and resistance in one of the most difficult tumors to treat, lung squamous cell carcinoma. We have the unique opportunity to utilize a genetic mouse model that accurately mimics human disease in combination with a state-of-the-art immunotherapeutic for lung cancer patients. It is not known why some patients do not respond to this immunotherapy or who will develop resistant disease. Our data to date suggest that resistance or lack of response to immunotherapy may be due to failure to effectively target cancer stem cells. Our next steps will be to perform gene expression studies to determine how cancer stem cells change when immunotherapy is not successful. These studies will yield cogent design and deployment of clinical trials with the best rational combination of agents for these acquired resistant and non-responsive lung cancer patients.

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

Antonella Dost, the graduate student performing the experiments described in this study, had significant training and professional development under this project. Antonella developed skills in the isolation of lung

cancer stem cells, mouse models of lung cancer, cell sorting, organoid culture techniques, and histological analysis. She has developed professional skills such as engaging with collaborators (by working with our collaborators in the Kwok Wong Lab), presenting her data in our lab meetings, and through the planning, execution and interpretation of the research described.

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?

NGFR will be tested as a CSC marker in primary xenografted human SCC (patient-derived xenograft or PDX model). We will first utilize 6 PDX samples from the Van Andel Research Institute from which we previously characterized frozen cell populations and identified a population of EpCAM positive tumor cells expressing high levels of NGFR. From these lung SCC PDX growing in mice, cells will be dissociated and NGFR+ and NGFR- populations will be isolated. Briefly, hematopoietic, endothelial, and immune cells will be excluded from the human tumor samples using CD45, CD31, and CD11b, respectively. The human tumors will also be stained and sorted for EpCAM to ensure that the NGFR+ and NGFR- populations are of human epithelial origin. Tumor cell injections will be done with the FACS isolated tumor cell populations in limiting dilution (e.g., 100 cells x 3 mice; 1000 cells x 3 mice; etc for NGFR+ or NGFR- cells) into NOD/SCID-IL2 receptor gamma chain knockout mice (NSG). Injected mice will be monitored by MRI for tumor development and resulting tumors will be analyzed by histology and FACS. The ability of each cell subpopulation to form lung tumors in recipients will be determined by histopathological analysis. Thoracic lymph nodes and distant organs including the liver, adrenal glands, brain, etc. will be collected from these recipients for histology to detect metastatic lesions. Tumors arising from injected cells, referred to as secondary tumors, will be analyzed for SCC markers including p63, SOX2 and NGFR. Secondary tumor cells will be fractionated by FACS and the NGFR+/- populations will be used in transplantation to determine their ability to form tertiary tumors, indicative of self-renewal. CSCs will be identified as the cell population with the most efficient tumor formation over multiple passages in vivo. This will require 108 NSG mice (3 cell dilutions in triplicate, 2 populations, 3 independent experiments). Differences in tumor propagation between test populations will be compared by t-test. Limiting dilution studies will be analyzed using L-Stat to calculate the frequency of CSCs.

In parallel with in vivo transplantation assays, the same sorted cell populations will be plated in 3D organoid culture as a complimentary readout of tumor-propagating activity. Importantly, we showed with murine *Lkb1/Pten* SCC and adenocarcinoma CSCs, that organoid co-cultures accurately predict which lung tumor cell populations have tumor-propagating activity in vivo^{6,7,14}. Equal numbers of each cell population will be cultured with Matrigel in transwells in combination with tumor stroma including hematopoietic and endothelial cells. After two weeks, the number of organoids arising from each population will be compared. Cell cultures will be fixed and paraffin embedded for immunohistochemical analyses to determine tumor cell differentiation status. The number of basal-like organoids exhibiting staining for p63 and other basal cell markers will be compared from each population (e.g. NGFR+/-) from at least 3 independent experiments for each PDX sample and analyzed by t-test. To functionally test the self-renewal abilities of these organoid forming populations, cultures will be dissociated, FACS sorted for NGFR again and replated for secondary organoid formation assays. If a population contains CSCs that can produce SCC, we expect to observe organoids with p63 staining derived from that sorted population.

To identify possible mediators of CSC activity we will prepare RNA from the CSC samples we have already acquired from *Lkb1/Pten* SCC and the non-CSC fraction of tumors and perform RNA-Seq. Similarly, if we find a functional CSC enriched population in the PDX, we will likewise isolate these CSC enriched and depleted cell populations and RNA will be prepared. RNA-Seq will be performed using the Illumina platform at the BCH Microarray Facility. Gene expression profiles of NGFR+ and NGFR- cells from SCC will be compared to learn the pathways governing CSC activity, including possible immune-escape pathways. We previously generated RNA-seq data from Sca1 and NGFR fractions of *Lkb1/Pten* tumors to which we can compare enriched pathways. This will require 60 mice (10 mice for CSC isolation, triplicate, 2 conditions).

Bioinformatic analyses will be focused on identifying transcripts that are significantly increased in CSCs compared to non-CSCs. We will use the RNA-seq pipeline in routine use in Peter Hammerman's group developed at the Broad Institute. Briefly cDNA libraries for sequencing will be prepared from total RNA using the Nugen Ovation system and quality controlled libraries sequenced to a minimum depth of 30 million reads. Sequenced libraries will be aligned and analyzed using the Prada pipeline, which has been previously used for TCGA projects as well as our own work using mouse models. This pipeline outputs transcript abundance (Fragments Per Kilobase of exon model per Million mapped reads) as both gene- and isoform-level expression. Differential expression analyses will be performed using the Genepattern suite as well as complementary tools including DESeq, GOSeq, DEGSeq, and BaySeq. Expression analyses will be carried across both mouse and human RNAseq data to identify common transcripts upregulated or downregulated in CSCs versus the non-CSC cell populations. We will select twenty genes that show enriched gene expression in murine and human CSCs versus non-CSCs for qPCR to validate expression differences. Importantly, the primary goal of these studies is to identify pathways/functions upon which SCC relies in order to better predict therapeutic approaches. We expect that factors dictating responses to the immune microenvironment will be highly expressed in CSCs.

Once key gene expression patterns shared between murine and human SCC CSCs are identified, we will examine the importance of candidate genes in CSC activity using blocking antibodies, knockdown or CRISPR strategies coupled with transplantation. As a proof-of-principle, we will first test the candidate gene NGFR. While NGFR is a marker of murine SCC CSCs, it may also have a functional role in CSC activity. We have previously shown that CD24, a marker of CSCs in the *Kras/p53* model of adenocarcinoma, is also required for tumor propagation and metastatic activity⁷. To determine if NGFR function is critical in SCC growth and progression, we will knock down NGFR in murine and human SCC cell lines and use ability to form colonies in 3D organoid cultures and in transplantation experiments to assess CSC activity. We have established several cell lines from *Lkb1/Pten* SCC that formed SCC after transplantation in vivo (not shown). These lines express NGFR, as do several human SCC cell lines that are readily available (H520, HCC95, data not shown). NGFR will be knocked down by infection of cells with lentiviral shRNA targeting NGFR. Knockdown will be determined by qPCR analysis and FACS when possible (e.g. for surface proteins such as NGFR) in cultured cell lines prior to in vivo injection as well as in resulting tumor specimens. shNGFR and shGFP (control) cells will be compared in 3D culture and in vivo assays as described above. As above, histopathological analyses and imaging studies will be employed. We have successfully knocked down NGFR in a human lung SCC cell line H520, and preliminary experiments show that cell populations with reduced NGFR are less fit to form tumor cell organoids than shGFP control cells in cultures (not shown). These data suggest that NGFR plays a role in CSCs beyond serving as a marker. Other candidate genes from the gene expression studies will be tested in a similar way as NGFR, and this work will define a set of genes and their respective pathways upon which SCC rely to propagate tumors. These genes will be potential targets for SCC therapeutic intervention.

4. IMPACT

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

These studies will uncover mechanisms of tumor maintenance, therapy response, and resistance in one of the most difficult tumors to treat, lung squamous cell carcinoma. We have the unique opportunity to utilize a genetic mouse model that accurately mimics human disease in combination with a state-of-the art immunotherapeutic for lung cancer patients. It is not known why some patients do not respond to this immunotherapy or who will develop resistant disease. These studies will yield cogent design and deployment of clinical trials with the best rational combination of agents for these acquired resistant and non-responsive lung cancer patients.

We have identified the first bona fide CSC population in lung SCC, and we now seek to characterize CSCs in human lung SCC. CSCs are often rare populations within tumors, and are not well represented in genomic gene expression analyses from whole tumor samples. Thus, standard tumor gene expression analyses have likely missed therapeutic opportunities that we can uncover studying CSCs. We will define murine and human

lung SCC that are resistant to immunotherapy, so that we can rationally design combination studies that will enhance the activity and duration of response to PD1/PD-L1 blockade. Our pre-clinical model is superior to performing combination immunotherapy studies only in patients.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

This project is providing significant professional development for a graduate student, resulting in increased productivity in our society.

5. CHANGES/PROBLEMS

Changes in approach and reasons for change

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report.

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

6. PRODUCTS

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Antonella Dost, Graduate Student

Researcher Identifier

Nearest person month worked: 12

Contribution to the Project: Antonella Dost has planned, executed and interpreted the results of all the experiments described.

Carla Kim, PI

Researcher Identifier

Nearest person month worked: 1

Contribution to the Project: Dr. Kim has planned and interpreted the results of all the experiments described. She has completed grant administration.

Kwok Wong, Co-PI

Researcher Identifier

Nearest person month worked: 1

Contribution to the Project: Dr. Wong has planned and interpreted the results of all the experiments described.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Changes in the active other support for Dr. Kim:

Closed:

U01HL100402- (Scadden)

Microenvironmental Control of Progenitors in Organ Dysfunction and Repair

The major goals of this project are aimed to identify, isolate, and characterize stromal cell types in hematopoietic, lung, and cardiac tissues and to determine the microenvironmental molecules that regulate endogenous stem cells from all of these tissues.

Dana Farber Cancer Institute

Modeling Cell Fate Transitions in Lung Cancer Stem Cells (Co-PI)

By discovering and dissecting the regulatory networks, we will find key factors that drive tumor development and cell fate decisions.

Newly Active:

Cystic Fibrosis Foundation Therapeutics *Molecular mechanisms limiting progression in cystic fibrosis*

Aim 1. To differentiate extreme CF patient iPS cells into airway epithelial cells for RNAseq and channelopathy analyses.

Aim 2. Human iPS cells differentiated into definitive lung endoderm will be co-cultured with liver endothelial cells in our 3D organoid assay and assessed for airway-specific differentiation.

MIT bridge

Designing treatments for SMARCA4/BRG1-mutant lung cancers

Aim 1. Engineer isogenic human and murine BRG1 mutant cell lines using CRISPR-Cas9 to dissect the mechanisms behind the sensitivity to combined EZH2 inhibition and etoposide treatment.

Aim 2. Perform preclinical studies of combined EZH2 inhibition and etoposide in vivo using autochthonous Kras driven GEMMs of lung adenocarcinoma with or without Brg1.

Aim 3. Establish combined EZH2 inhibition and etoposide as a candidate therapeutic regimen for SMARCA4 mutant human lung adenocarcinoma.

1R01H132266-01

Mechanisms of Thrombospondin-1 as a pulmonary vascular mediator

We hypothesize that endothelial-derived thrombospondin-1 induced by BMP4-NFATc1 signaling inhibits pulmonary vascular development and is required for proper lung development.

Aim 1. Determine how Tsp1-CD47 interactions regulate vasculogenesis and lung development using organoid cultures and mouse knockout strains

Aim 2. Define the mechanisms by which Tsp1-dependent Rspo2 mediates pulmonary vascular development by evaluating ligand response in vitro and after hyperoxic injury in vivo

Dr. Wong relocated his laboratory to NYU. His role on the project remains the same.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report.

9. APPENDICES

Nothing to report.