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TITLE:
Targeting of Cancer Stem Cells and Their Microenvironment in Early-Stage Mutant
K-ras Lung Cancer

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
The overall goal of this proposal is to understand the role of the Hh pathway in the maintenance and proliferation of lung adenocarcinoma progenitor cells and to establish therapeutic strategies that target the stem cell and its microenvironment for early stage lung cancer. We have completed the analysis of DHH expression in lung cancer cell lines, complementing previous findings of SHH and IHH. Using anti-SHH/IHH antibody 5E1 conjugated with AlexaFluor 647, we have successfully identified a NOTCH3+SHH+ cell population that has characteristics of stem-like features. We have also tested inhibition of paracrine Hh pathway using both murine embryonic fibroblasts and normal murine lung fibroblasts. Whereas tumor growth is dependent on paracrine Hh pathway of MEFS, they are much less dependent on paracrine Hh pathway of lung fibroblasts. Analysis of potential Hh pathway targets of these fibroblasts showed that fibroblasts respond differently in response to SHH. We have completed the generation of $LSL\text{-}Kras^{G12D/+};Trp53^{fl/fl};R26^{luc/+}$ mouse strain to test for tumor growth and metastasis. We have also generated orthotopic mouse models of lung adenocarcinoma cells with H23-FLG that express high levels of SHH. We will use these models to further pursue studies for the prevention of cancer metastasis.

15. SUBJECT TERMS
Non-small cell lung cancer, cancer stem cells, Hedgehog pathway, metastasis, tumor epithelial-stromal interactions

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Table of Contents

INTRODUCTION:

Lung cancer accounts for the greatest number of cancer deaths in the U.S. and the world. Five-year survival rates are <50% and ~30% for patients with stage I and II non-small cell lung cancer, respectively, despite surgery and adjuvant chemotherapy, in large part due to recurrence at the original site of disease and metastasis to distant sites. Cancer stem cells have been proposed to be the cancer cells that metastasize to other areas of the body and are resistant to chemotherapies. We have found that in mutant K-ras lung cancers, a group that comprises ~30% of NSCLC with no available targeted agents, only a subset of tumor cells have mutant K-ras activity. These cells also express high levels of secreted SHH ligand implying that mutant K-ras may be active in a cancer stem cell compartment and, analogous to lung development, use paracrine stromal Hh pathway activity to promote its growth and metastases. The _overall goal_ of this proposal is to understand the role of the Hh pathway in the maintenance and proliferation of mutant K-ras lung cancer progenitor cells and to establish therapeutic strategies that target the stem cell and its microenvironment for early stage lung cancer. Therapies that target cancer stem cells and its supportive environment may prevent recurrences after treatment and possibly lead to a cure for early stage lung cancer.

Figure 1. Expression of Hh ligands in human lung adenocarcinoma cell lines. mRNA of Hh ligands, SHH (A), IHH (B), and DHH (C) was measured in lung adenocarcinoma by qPCR.

KEYWORDS:

Non-small cell lung cancer
cancer stem cells
Hedgehog pathway
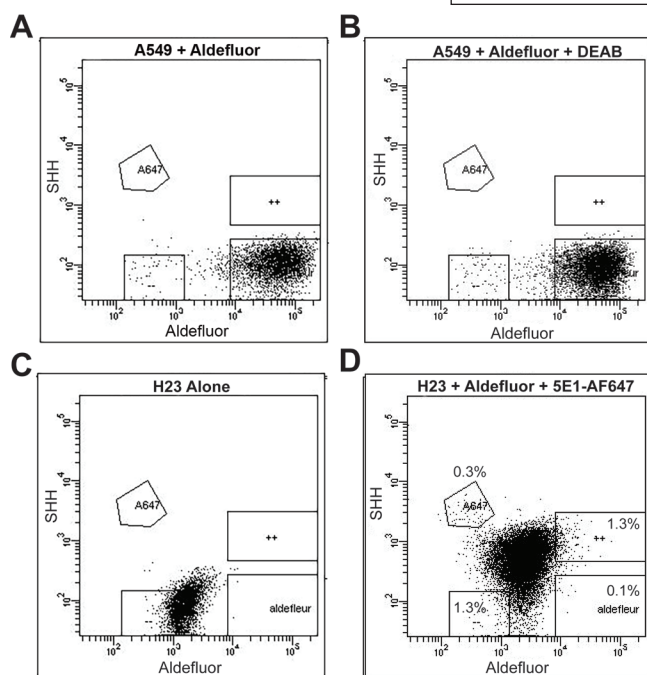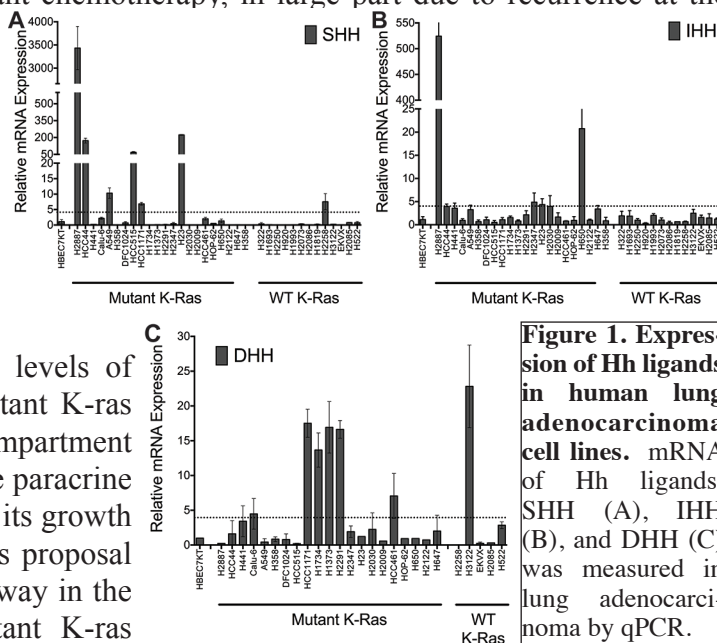metastasis
tumor epithelial-stromal interactions

ACCOMPLISHMENTS:

I. Major Goals of the Project:

AIM 1. To determine that cells expressing SHH are the CSCs and that stromal Hh pathway is critical for tumor growth in mutant K-ras lung cancer. Months 1-18

Goal 1. Isolation and testing of SHH-expressing lung cancer cells for stem cell capacity

Goal 2. Isolation of ALDH$^+$ cells and

Figure 2. FACS sort of H23 cancer cells using Aldefluor assay. Images of FACS-analyses. (A) A549 control lung cancer cells were incubated with Aldefluor reagent. (B) A549 control lung cancer cells were incubated with Aldefluor regent and DEAB, an inhibitor of aldehyde dehydrogenase. (C) High SHH-expressing H23 with no Aldefluor or 5E1-AF647 were FACS analyzed. (D) High SHH-expressing H2887 cells were incubated with Aldefluor and 5E1-AF647 antibody.

testing for SHH-expression and stem cell capacity

       Goal 3. Test of ALDH⁺SHH+ cells for stem cell capacity

AIM 2. To test drug combinations that target pathways active in tumor epithelium and the Hh pathway in stroma. Months 13-24.

       Goal 1. Combination drug therapy targeting tumor epithelial and stromal Hh pathway active cells in vitro.

       Goal 2. Combination drug therapy targeting tumor epithelial and stromal Hh pathway to inhibit metastases of lung cancer stem cells in vivo.

II. Accomplishments under Goals:

For Aim 1, in addition to SHH and IHH that we had shown previously (Fig. 1A, B), we have also measured DHH ligand expression in a number of human lung cancer lines (Fig. 1C) to complete the survey of Hh ligand expression in human lung adenocarcinoma cell lines. We have also continued with our work to further identify whether SHH-expressing cells in lung adenocarcinoma may act as the stem cell. As noted in the past progress report, SHH+ did not show any increase in liquid colony formation or in cell proliferation compared to SHH- cells. Therefore, we turned to identify aldehyde dehydrogenase (ALDH) positive cells as they have been reported to contain a stem cell population (1) (Aim1, Goal 2). We used the Aldefluor assay (StemCell Technologies) in which a green fluorescent BODIPY moiety is linked to aminoacetaldehyde, an aldehyde dehydrogenase substrate, and thus, cells expressing ALDH can be identified by flow cytometry or FACS-sorted. We used A549 lung cancer cells as positive controls as recommended by manufacturer due to its high expression of ALDH. Indeed, we see increased number of green fluorescent cells (Fig. 2A) indicative of high ALDH
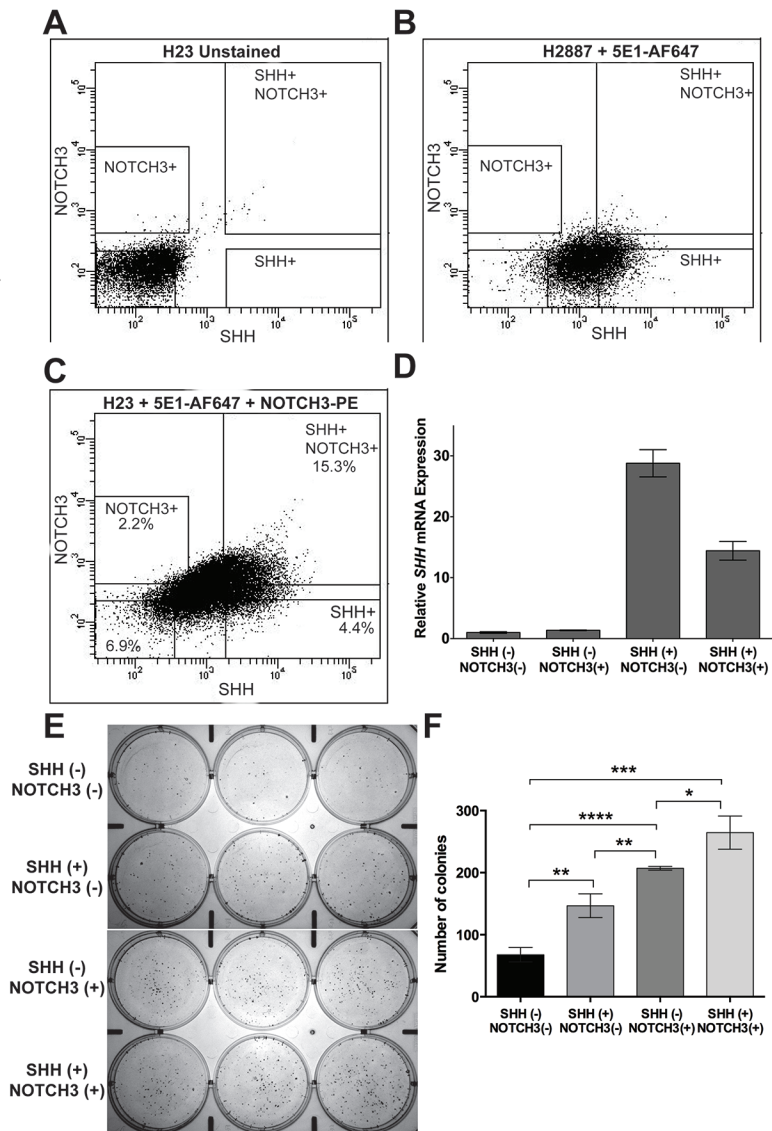

Figure 3. FACS sort of H23 cancer cells by NOTCH3. Flow cytometry experiments are shown for (A-C). (A) H23 cells without any antibody incubation. (B) H2887 cells incubated with 5E1-AF647 is shown. (C) FACS-sort of H23 cells incubated with NOTCH3-PE and 5E1-AF647. Percentage of cells within each gate is shown. (D) SHH mRNA expression relative to SHH(-) NOTCH3(-) cells as measured by qPCR. (E) Liquid colony formation assay of H23 cells FACS-sorted as in panel C. (F) Quantification of liquid colony formation assay shown in panel E. * $p < 0.03$, ** $p < 0.006$, .*** $p < 0.0005$, *** $p < 0.0001$.

expression. However, treatment with DEAB, an ALDH antagonist, had no effect in inhibiting the green fluorescence of the Aldefluor assay (Fig. 2B). We discussed these issues with the technical staff of StemCell Technologies, the manufacturer of the Aldefluor assay. They suggested several changes to the protocol, including (1) raising the amount of DEAB to 3x the suggested dose (please note that the protocol for the assay does not give the concentration of DEAB), (2) pretreating the cells with DEAB prior to incubation with the Aldefluor substrate and (3) combination of both suggestions 1 and 2. We performed these experiments and did not see any downward shifts in green fluorescence (data not shown). We received a new lot of DEAB from StemCell Technologies and repeated the experiments again by pretreating A549 cells with the high DEAB dose. Again, we failed to see any diminishment of the Aldefluor green fluorescence with the new lot of DEAB (data not shown). However, these studies did give us the flow cytometry gates to identify ALDH+ cells. We decided to move forward to FACS-sort high SHH-expressing H23 cells using Aldefluor and 5E1-AF647, an anti-SHH/IHH monoclonal antibody that we conjugated with the fluorescent dye Alexafluor-647 using Zenon labeling kit (Thermofisher). Flow cytometry of unstained H23 cells were used to set the SHH+ gate (Fig. 2C). We were able to sort for SHH+Aldefluor+ (1.3%), SHH+Aldefluor- (0.3%), SHH-Aldefluor+ (0.1%), SHH-Aldefluor- (1.3%) (Fig. 2D). We attempted to perform a liquid colony formation assay but viability of sorted cells were poor (<20%) and those that did survive died once in culture. We had similar problems with previous attempts at FACS-sorting our cells. After further investigation and discussions with other groups more experienced in FACS-sorting of epithelial cells, we identified the problem as the sheath pressure being too high during the sorting. We lowered the sheath pressure significantly and also switched to a 130 micron nozzle, the largest sized nozzle on the flow cytometer, to minimize any trauma to the cells. To further aid the viability of cells, we added HEPES 2.5 mM and 3% FBS to solution in tube receiving the FACS-sorted cells to stabilize
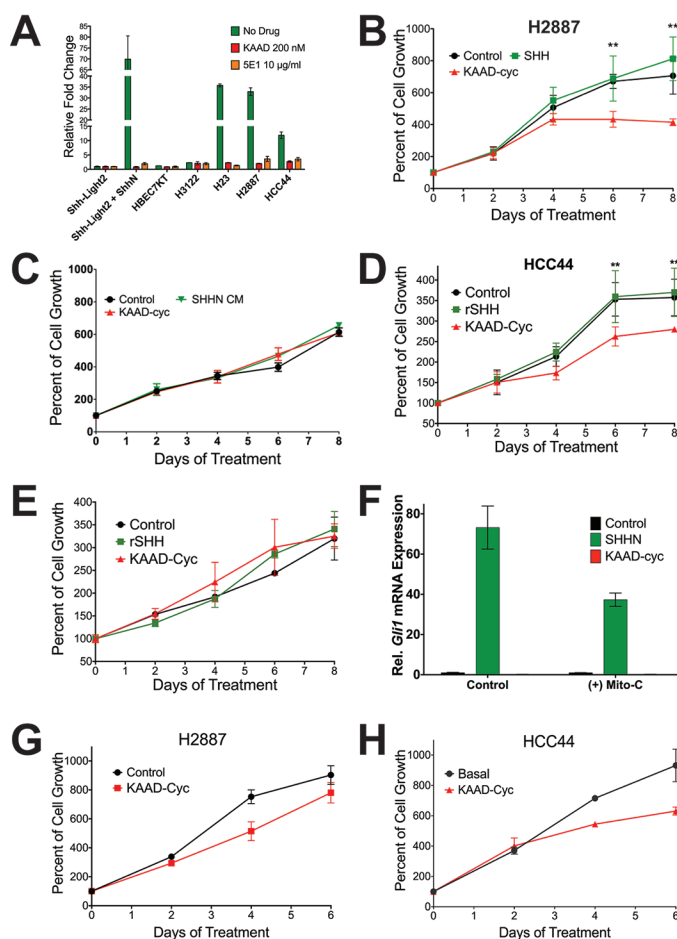

Figure 4. Dependence of paracrine Hh pathway for cancer cell growth. (A) Co-culture of high SHH-expressing H23, H2887 and HCC44 and low SHH-expressing H3122 cancer cell and HBEC7kt with Shh-Light2 reporter cell line activate the Hh pathway, which is inhibited by KAAD-cyclopamine (SMO antagonist) and 5E1 anti-SHH/IHH monoclonal antibody. (B-C) Growth of high SHH-expressing H2887-FLG cells co-cultured with (B) NIH-3T3 fibroblasts or (C) alone. (D-E) Growth of high SHH-expressing HCC44-FLG cells co-cultured with (D) NIH-3T3 fibroblasts or (E) alone. (F) Hh-pathway activation as measured by GLI1 mRNA expression was measured by qPCR. CCL-206, with and without mitomycin-C pretreatment 10 µg/ml for 3 hours, was incubated with basal conditioned medium (CM), SHHN CM, or SHHN CM with KAAD-cyclopamine 250 nM. (G-H) Growth assays of (G) H2887-FLG and (H) HCC44-FLG co-cultured with mitomycin-c pretreated CCL-206 normal murine lung fibroblasts and treated with vehicle control or KAAD-cyclopamine 300 nM. .

the pH and enrich the cell solution, respectively. Subsequent test experiments showed that these changes increased the viability of post-FACS-sorted cells to >80%.

Once the technical issues of FACS-sorting were resolved, we decided to move forward with NOTCH3+ instead of ALDH as a marker for several reasons. NOTCH3 is expressed on ALDH+ lung cancer stem cells (1) and one of the key markers of mutant $KRAS^{G12D}$ murine lung cancer stem cells (2). Furthermore, the lack of DEAB's effect in the Aldefluor assay (Fig. 2B) made us wary whether we were truly able to capture ALDH- cells. We decided to focus on H23 lung cancer cells due to better characteristics in vitro and in vivo than H2887 cells. Unstained H23 cells were used to establish the NOTCH3 positive and negative flow cytometry gates (Fig. 3A). H2887 was used to set the gates for SHH (Fig. 3B). Here, we decreased the size of the gates to obtain cells that were more likely to have positive and negative SHH cells given our previous experiences that some SHH- cells may have been positive due to the secretion of SHH protein. Thus, smaller gate sizes biased toward higher and lower fluorescence will more likely capture true SHH+ and SHH- cells, respectively. We successfully FACS-sorted H23 cells stained with 5E1-AF647 and NOTCH3-PE with the gates noted above into SHH+NOTCH3+ (15.3%), SHH+NOTCH3- (4.4%),
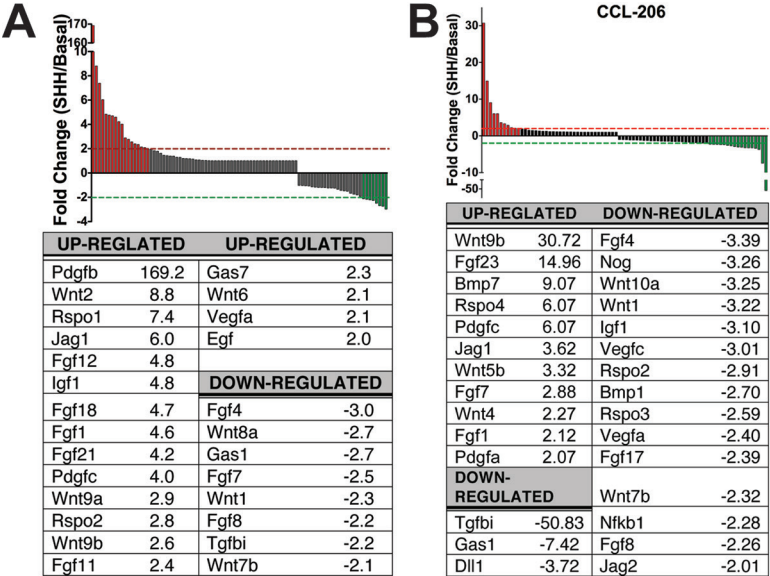

Figure 5. Response of NIH-3T3 and CCL-206 fibroblasts in response to SHH. (A) mRNA expression of ligands in NIH-3T3 cells stimulated with rSHHN 1 µg/ml. Dashed lines represent 2-fold upregulation (red) or downregulation (green) and detailed in table below. (B) mRNA expression of ligands in CCL-206 lung fibroblasts stimulated with rSHHN 1 µg/ml. Dashed lines represent 2-fold upregulation (red) or downregulation (green) and detailed in table below.
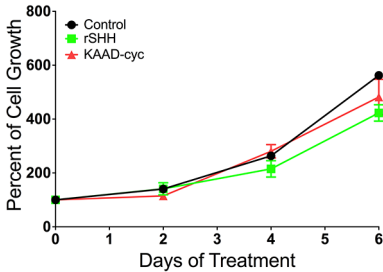
SHH-NOTCH3+ (2.2%), and SHH-NOTCH3- (6.9%) (Fig. 3C). Evaluation of SHH mRNA of the sorted cell populations by quantitative PCR (qPCR) showed increased SHH mRNA expression in the SHH+ cell populations sorted by 5E1-AF647 (Fig. 3D), thus resolving an issue we noted in the first progress report where SHH-expression did not correlate with SHH+ or SHH- cells. To test for cancer stem- or progenitor-like properties, we performed dilution limiting-liquid colony formation assays with the FACS-sorted H23 cell populations. One thousand cells per well of each FACS-sorted H23 cell population were plated in in triplicate in 6 well plates. NOTCH3+SHH+ had the greatest number of colonies, followed by NOTCH3+SHH-, NOTCH3-SHH+, and NOTCH3-SHH- cells (Fig. 3E, F). We also injected these four cell populations subcutaneously into the flanks of 5 NOD-SCID-IL2Rγ$^{-/-}$ mice with cell doses of 10^2, 10^3, and 10^4 for each cell population to test for

Figure 6. HCC515-FLG cancer cells do not depend on paracrine Hh pathway for cancer cell growth. High SHH-expressing HCC515-FLG cells were co-cultured with NIH-3T3 cells pretreated with mitomycin-C and treated wtih rSHHN 1 µg/ml or KAAD-cyclopamine 300 nM. Tumor cell growth monitored by firefly luciferase expression.

7

growth *in vivo*. We did not see any tumor growth in nearly 4 months. We have tested the H23 cells and they do grow *in vivo* both subcutaneously (data not shown) and orthotopically (Fig. 7B) when 10^6 cells are injected, albeit slowly. We will repeat this experiment using greater number of FACS-sorted cells.

Our *in vitro* results suggest that NOTCH3 is a more reliable marker of stem-like properties than SHH alone (Fig. 3E, F). However, the combination of NOTCH3 and SHH shows even more promise as a more refined cell population with stem cell-like properties that will need to be further tested in other cancer cell lines such as H2887 and HCC44.

For Aim 2, as we were still in the process of finding the correct antigens to identify cell populations with stem cell-like features (see results above), we continued with bulk cell lines and their interactions with stroma. We had previously shown that high SHH-expressing lung adenocarcinoma cell lines H2887, HCC44, and H23 induce the Hh-pathway when

Figure 7. H23-FLG lung cancer cell line. (A) H23 lung adenocarcinoma cell line stably expressing firefly luciferase and GFP (not shown). Firefly luciferase is expressed in a linear manner with increasing cell numbers that range over 2 orders of magnitude. (B) Representative bioluminescence imaging (BLI) of an H23-FLG orthotopic xenograft 5 weeks after injection of 1 million cells. BLI exposure is 10 seconds. .(C) Firefly luciferase increases linearly with increasing cell numbers of H2887-FLG. (D) Representative BLI of mouse injected with 1 million H2887-FLG cells 8 weeks after injection in left lung. Exposure is 60 seconds. The BLI detected on the paws are non-specific due to the high exposure time.

co-cultured with SHH-Light2 cells, a clonal NIH-3T3 based cell line with an 8x-GLI-luciferase reporter (3) (Fig. 4A). Inhibition of stromal Hh pathway by KAAD-cyclopamine 200 nM, a potent SMO inhibitor, and 5E1 10 µg/ml, a potent anti-SHH/IHH blocking antibody (4), suggests that SHH from cancer cells act on the fibroblast SHH-Light2 cells in a paracrine manner (Fig. 4A). We also previously showed that growth of H2887-FLG cells that stably express firefly luciferase and GFP was adversely affected by inhibition of Hh pathway activation in NIH-3T3 cells, pre-treated with mitomycin-c to inhibit growth of stromal cells, when treated with KAAD-cyclopamine 300 nM (Fig. 4B). Since then, H2887-FLG cells alone treated with KAAD-cyclopamine 300 nM did not affect tumor cell growth (Fig. 4C) suggesting that tumor cell proliferation does not depend on autocrine Hh pathway. Similarly, growth of HCC44-FLG cells co-cultured with NIH3T3-cells with mitomycin-c was inhibited by treatment with KAAD-cyclopamine 300 nM (Fig. 4D) whereas growth of HCC44-FLG cells alone were not affected by KAAD-cyclopamine (Fig. 4E). These results suggest that SHH activates stromal cells in a paracrine manner rather autocrine activation of the Hh pathway in tumor cells. As NIH-3T3 fibroblasts are embryonic fibroblasts, their character and responses to Hh ligands may be different than those of lung fibroblasts that actively interact with lung cancer cells. CCL-206 murine lung fibroblast cell line (5) was developed from neonatal murine lungs, respond to SHHN conditioned medium (CM), and pathway activation is inhibited by KAAD-cyclopamine 250 nM (Fig. F). As we have used mitomycin-c to inhibit fibroblast growth in co-culture assays of tumor growth (Fig. 4B, D), we tested the effect of Hh pathway activation on CCL-206 cells pretreated with mitomycin-C 10 µg/ml for 3 hours, washed, and then incubated
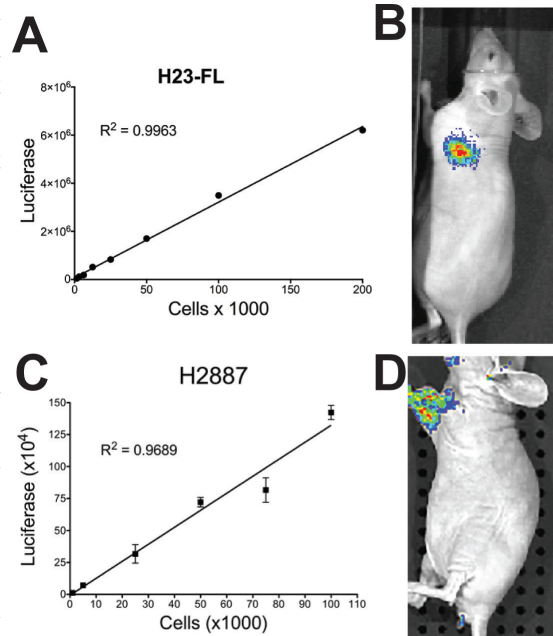
with basal CM, SHHN CM, and SHH CM + KAAD-cyclopamine 250 nM. Hh-pathway was robustly induced, as measured by GLI1 mRNA expression, with SHHN CM – albeit less than without mitomycin-c, and treatment with KAAD-cyclopamine suppressed pathway activation (Fig. 4F) suggesting that CCL-206 lung fibroblasts pre-treated with mitomycin-c maintained Hh pathway responsiveness. Analogous to co-culture growth experiments performed with NIH-3T3 cells (Fig. 4B, D), we tested the growth of H2887-FLG and HCC44-FLG cells co-cultured with CCL-206 normal lung fibroblasts pre-treated with mitomycin-c. The growth inhibitory effect by KAAD-cyclopamine 300 nM seen with NIH-3T3 embryonic fibroblasts (Fig. 4B, D) was severely blunted in the growth assays with CCL-206 lung fibroblasts (Fig. 4G, H). These results suggest that the responses of lung fibroblasts to Hh pathway stimulation are not only different than those of embryonic fibroblasts, but the secreted stromal factors from lung fibroblasts do not significantly impact tumor growth as embryonic fibroblasts. These results were unexpected and most likely reflect the different transcriptional programs of lung fibroblasts compared to embryonic fibroblasts. Thus, we examined the potential factors secreted from NIH-3T3 and CCL-206 fibroblasts. NIH-3T3 fibroblasts were treated with and without recombinant SHHN (rSHHN, R&D) 1 µg/ml and resultant total mRNA tested on custom qPCR array plates (Bio-Rad) consisting of secreted ligands that may be target genes of Hh pathway activation including all ligands for Wnt, Egf, Fgf, Igf, Bmp, Tgfβ, Notch, Gas, Nfκb, Pdgf, Rspo, and Vegf pathways (Fig. 5A). Analogously, CCL-206 cells treated with and without rSHHN were also tested with the custom qPCR array plate (Fig. 5B). Several differences are noted. First, more genes are upregulated in NIH-3T3 fibroblasts than CCL-206 fibroblasts in response to SHH. Conversely, more genes are downregulated in CCL-206 cells than in NIH-3T3 cells. Secondly, although the upregulated genes in both cell types have common pathways such as Wnt and FGF, the specific ligands are different and may have differential roles analogous to Wnt2 and Wnt7b in lung airway development (6). The differential expression of these genes may explain the loss of cancer cell proliferation in our assays and will be further pursued.

In the previous progress report, we showed that high SHH-expressing HCC515-FLG lung adenocarcinoma cells, stably expressing firefly luciferase, can establish as an orthotopic xenograft tumor and readily metastasizes. HCC515-FLG cells express high levels of SHH mRNA (Fig. 1A). Therefore, we tested the dependence of *in vitro* growth of these cells co-cultured with NIH-3T3 fibroblasts analogous to the experiments in Fig. 4B and D. Surprisingly, inhibition of fibroblast SHH-pathway activation by KAAD-cyclopamine did not have any effect on the growth HCC515-FLG cancer cells (Fig. 6). The etiology of these results are unclear and further experiments will be needed to elucidate the mechanisms of these results. We have generated another lung cancer cell line that stably expresses firefly luciferase and GFP, H23-FLG (Fig. 7A). We showed that native H23 cells can induce the Hh pathway in Hh-responsive fibroblasts (Fig. 4A). We also confirmed that the H23-FLG cells can indeed form orthotopic xenograft tumors in nude mice (Fig. 7B) as determined by bioluminescence imaging (BLI) albeit slowly. Injection of 1 million cells took 5 weeks to show a moderate signal (Fig. 7B). We will repeat the experiments with H23-FLG orthotopic xenografts to see if the tumors will metastasize. We have also made H2887-FLG cell line (Fig. 7C) whose growth is dependent on paracrine Hh pathway signaling (Fig. 4B). However, these cells cannot establish an orthotopic transplant when injected with 1 million cells (Fig. 7D), up to 8 weeks.

We have completed the generation of *LSL-Kras$^{G12D/+}$;Trp53$^{fl/fl}$;R26R$^{luc/+}$* mouse strain for the experiments in Aim 2. Before initiating the proposed experiments in Aim 2, we examined the

incidence of metastases in control *LSL-Kras$^{G12D/+}$;Trp53$^{fl/fl}$* mice in other projects. Only 1 of 9 mice showed any metastasis to mediastinal lymph nodes and none to chest wall or other distant organs 10 weeks after tumor initiation. For this project, we proposed assessing metastases by bioluminescence at 7 weeks after tumor initiation with adenovirus-expressing-cre recombinase (adeno-cre). Instead, we will use the proposed secondary time point of 16 weeks to determine if metastases are present in these mice in a much greater proportion.

III. Opportunities for training and professional development this project has provided
1. Formal and informal one-on-one meetings with mentors Dr. John Minna and Dr. Joan Schiller. Please note that Dr. Schiller left UTSW in Jan. 2016 to start a position at another institution. Dr. Minna has remained throughout the grant period.
2. Faculty mentor meetings with Faculty Mentor Committee consisting of Dr. John Minna (Professor), Dr. Michael White (Professor), and Dr. Rolf Brekken (Professor) for guidance on all aspects of science and career development. Dr. Michael White left UTSW in summer of 2016 and has been replaced by Dr. Lawrence Lum.
3. Monthly UTSW-MD Anderson Lung Cancer SPORE video conference meeting.
4. Bi-weekly UTSW Lung Cancer Disease-Oriented Team meetings.
5. Weekly clinical Thoracic Oncology Tumor Board meetings.

IV. How were results disseminated to communities of interest?
The results of our studies are still preliminary and have not been disseminated.

V. What do you plan to do during the next reporting period to accomplish the goals?
Nothing to report – this is the final reporting period.

IMPACT:
I. Impact on development of the principle discipline of the project: A chief hypothesis for this project is that abrogation of the stromal Hh pathway was critical for the growth and metastasis of the cancer stem cells and thus, decreases gross tumor growth. Our results thus far suggest that NOTCH3 is a marker of cells with stem-like features than SHH. However, the finding that the NOTCH3+SHH+ cell population has the greatest capacity to form colonies suggests that we have found markers that offer a greater enrichment of cancer-initiating cells. This will need to be further verified with other lung cancer cell lines and in autochthonous mouse model of mutant K-ras lung cancer to solidify our findings. Secondly, our results further suggest that fibroblasts from different tissues and different contexts respond differently to the same stimuli. Thus, context matters i.e. epithelial cells behave differently than fibroblasts and fibroblasts from different tissue contexts behave differently from one another. In addition, CCL-206 cells were derived from neonatal murine lungs. It may very well be that mature lung fibroblasts from adult mice may very well behave differently than CCL-206 cells. Thus, the common practice of testing for pathway response or protein expression with mouse embryonic fibroblasts in lieu of epithelial cells or even other fibroblasts should be considered with caution. Further investigation is required to delineate the differences between the fibroblast cell lines we have used here and how these cell lines may differ from endogenous murine lung fibroblasts.

II. Impact on other disciplines: Nothing to report. The data are preliminary and further experiments are required for firm conclusions.

III. Impact on technology transfer: Nothing to report

IV. Impact of society beyond science and technology: Nothing to report. The data are preliminary and further experiments are required for firm conclusions.

CHANGES/PROBLEMS:
I. Changes in approach and reasons for change.
No significant changes were made during this reporting period.

II. Actual or anticipated problems or delays and actions or plans to resolve them.
None

III. Changes that had a significant impact on expenditures.
None.

IV. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and select agents.
No significant changes were made during this reporting period. The orthotopic transplantation of lung cancer cells noted in Fig. 7 were part of the approved IACUC protocol reviewed by ACURO in 2014.

PRODUCTS:
No new products during this time period.

PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS:
I. Participants
Key Personnel

Name:	James Kim
Project Role:	Principal Investigator
Nearest Person Month Worked	6.0
Contribution to Project:	Planned, guided and execution of experiments of this proposal

Name:	John Minna
Project Role:	Other Significant Contributor
Nearest Person Month Worked	0.0
Contribution to Project:	Mentor and provision of cell lines and other reagents.

Name:	Lawrence Lum
Project Role:	Other Significant Contributor
Nearest Person Month Worked	0.0
Contribution to Project:	Lend expertise and reagents in cell biological techniques and Wnt pathway.

Non-Key Personnel

Name:	Baozhi Chen
Project Role:	Research Scientist
Nearest Person Month Worked	1.4
Contribution to Project:	Execution of cell biology and mouse experiments

Name: Sahba Kasiri	
Project Role: Postdoctoral Fellow	
Nearest Person Month Worked: 9.0	
Contribution to Project: Execution of cell biology and mouse experiments	
Funding Support: NIH T32 Postdoctoral Training Grant at UTSW	

Name:	Alexandra Wilson
Project Role:	Research Technician II
Nearest Person Month Worked	5.1
Contribution to Project:	Breeding and genotyping of mice, support and execution of mouse and cell culture experiments

Name:	Zhiqun Zeng
Project Role:	Research Associate
Nearest Person Month Worked	9.0
Contribution to Project:	Breeding and genotyping of mice, support and execution of mouse and cell culture experiments

Name:	Ummay Marriam
Project Role:	Research Technician II
Nearest Person Month Worked	3.3
Contribution to Project:	Breeding and genotyping of mice, support and execution of mouse and cell culture experiments

Please note that we had a transition of personnel of technicians and research associates who handle our mice. Alexandra Wilson started on this project but decided to leave UTSW for professional reasons. Zhiqun Zeng was hired to replace Ms. Wilson. However, Ms. Wilson overlapped with Ms. Zeng for ~3 month instead of the planned 1 month overlap due to a delay in the start of the next position for Ms. Wilson. During this overlap, Ms. Wilson trained Ms. Zeng and assisted with the experiments. Ms. Zeng subsequently left the lab in 2016 for another position at UTSW and Ms Ummay Marriam was hired to replace Ms. Zeng. They briefly overlapped for one week. Due to this brief overlap, Ms. Wilson was re-hired on a part-time basis to help train Ms. Marriam in breeding and genotyping of mice and assistance in the experiments.

II. Change in active support for PI.
A. New grants during this proposal period.

1. RSG-16-090-01-TBG (PI: James Kim)   07/01/2016 – 06/31/2020   2.4 cal months
   American Cancer Society
   "Disruption of Oncogenic Epithelial-Stromal Interactions"
   The major goal of this project is to elucidate the role of paracrine Hedgehog pathway in the growth and stimulation of lung cancer growth in the later stages of lung cancer.
   Role: PI

2. RP160089 (PI: Ralph Deberardinis)   03/01/2016 - 02/28/2019 (JK will started on 09/01/2016)
   1.2 cal months
   Cancer Prevention and Research Institute of Texas
   " Carbamoyl Phosphate Synthase-1: A new metabolic liability in lung cancers"
   The goal of this proposal is to identify the mechanism by which LKB1 regulates the expression of the urea cycle enzyme carbamylphosphate synthase-1 (CPS-1), a new metabolic vulnerability

in non-small cell lung cancers containing mutations in KRAS and LKB1, and to understand why KRAS/LKB1 co-mutant cells are dependent on CPS-1, in culture and in vivo
Role: Collaborator

7. 1R01CA196851 (PI: Lawrence Lum)   04/01/2016-03/31/2021   (JK   will   started   on   09/01/2016)
0.48 cal months
National Institutes of Health
"Targeting vismodegib-resistant tumors using BH3 mimetics"
The goal of this proposal is to evaluate the utility of BH3 mimetics in targeting drug resistant Hh-associated tumors
Role: Collaborator

B. Completed grants during this proposal period
1. SKF-14-057 (PI: James Kim)   7/01/2014 – 06/30/2015
2.4 cal months
The Sidney Kimmel Foundation for Cancer Research
 "The Interaction Of Stromal Hedgehog Pathway With Cancer Stem Cells Of Lung Adenocarcinoma"
The major goal is to characterize and develop antibody that specifically target stromal Hh pathway in lung adenocarcinoma but not in immune cells and develop therapeutic strategies with this antibody in combination with other therapeutics that target lung cancer stem cells.
Role: PI

No overlap in any listed proposals with this Dept. of Defense proposal.

III. Other Organization/Partners? Nothing to report.

SPECIAL REPORTING REQUIREMENTS: Not applicable

APPENDIX: References listed in the Annual Report

REFERENCES:
1.   Sullivan JP, Spinola M, Dodge M, Raso MG, Behrens C, Gao B, Schuster K, Shao C, Larsen JE, Sullivan LA, Honorio S, Xie Y, Scaglioni PP, DiMaio JM, Gazdar AF, Shay JW, Wistuba, II, Minna JD. Aldehyde dehydrogenase activity selects for lung adenocarcinoma stem cells dependent on notch signaling. Cancer Res. 2010;70(23):9937-48. Epub 2010/12/02. doi: 10.1158/0008-5472. CAN-10-0881. PubMed PMID: 21118965; PMCID: 3058307.

2.   Zheng Y, de la Cruz CC, Sayles LC, Alleyne-Chin C, Vaka D, Knaak TD, Bigos M, Xu Y, Hoang CD, Shrager JB, Fehling HJ, French D, Forrest W, Jiang Z, Carano RA, Barck KH, Jackson EL, Sweet-Cordero EA. A rare population of CD24(+)ITGB4(+)Notch(hi) cells drives tumor propagation in NSCLC and requires Notch3 for self-renewal. Cancer Cell. 2013;24(1):59-74. Epub 2013/07/13. doi: 10.1016/j.ccr.2013.05.021. PubMed PMID: 23845442.

3.   Taipale J, Chen JK, Cooper MK, Wang B, Mann RK, Milenkovic L, Scott MP, Beachy PA. Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine. Nature. 2000;406(6799):1005-9. Epub 2000/09/13. doi: 10.1038/35023008. PubMed PMID: 10984056.

4.   Ericson J, Morton S, Kawakami A, Roelink H, Jessell TM. Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity. Cell. 1996;87(4):661-73. Epub 1996/11/15. PubMed PMID: 8929535.

5.   Bermudez O, Hennen E, Koch I, Lindner M, Eickelberg O. Gli1 mediates lung cancer cell proliferation and Sonic Hedgehog-dependent mesenchymal cell activation. PLoS One. 2013;8(5):e63226. doi: 10.1371/journal.pone.0063226. PubMed PMID: 23667589; PMCID: 3646741.

6.   Morrisey EE, Hogan BL. Preparing for the first breath: genetic and cellular mechanisms in lung development. Dev Cell. 2010;18(1):8-23. Epub 2010/02/16. doi: S1534-5807(09)00527-9 [pii]10.1016/j.devcel.2009.12.010. PubMed PMID: 20152174.