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14. ABSTRACT

Invadopodia are actin-based cellular protrusions found in many invasive cancer cell types. Non small cell lung cancers (NSCLCs) are highly invasive and metastatic. The high mortality rate of NSCLC is attributable to the finding of overt metastases in the majori of patients at the time of diagnosis, as well as to the inoperable nature of many tumors. Yet remarkably, no studies have evaluate invadopodia formation and function in lung cancer cells. Unpublished immunohistochemical evidence from our laboratory suggest that the invadopodia scaffold protein Tks5 is expressed in the majority of primary NSCLCs, and to particularly high levels in at lea 25% of specimens, suggesting that invadopodia may play a role in NSCLC progression. The finding that some fraction or sub-type of NSCLC may use invadopodia as a mechanism of tumor growth and invasion may ultimately have clinical relevance. Our hypothesis is that invadopodia form in NSCLCs as a result of EGFr and KRas signaling and that this in turn promotes invasion an tumor cell growth. We will first evaluate the expression of invadopodia proteins in a panel of NSCLC lines previously phenotyped for EGFr and KRas status. Next we will determine the effect of invadopodia loss on invasion and tumor cell growth in vitro. Finally we will evaluate the role of invadopodia in tumor growth and metastasis in vivo. This focused yet comprehensive analysis will elucidate the frequency of invadopodia involvement in the invasive behavior of lung cancer cells, and if appropriate, set the stage for more in depth studies.

15. SUBJECT TERMS
Invadopodia, metastasis, invasion, KRas, EGF receptor

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Abstract:
Invadopodia are actin-based cellular protrusions found in many invasive cancer cell types. Non small cell lung cancers (NSCLCs) are highly invasive and metastatic. The high mortality rate of NSCLC is attributable to the finding of overt metastases in the majority of patients at the time of diagnosis, as well as to the inoperable nature of many tumors. Yet remarkably, no studies have evaluated invadopodia formation and function in lung cancer cells. Unpublished immunohistochemical evidence from our laboratory suggests that the invadopodia scaffold protein Tks5 is expressed in the majority of primary NSCLCs, and to particularly high levels in at least 25% of specimens, suggesting that invadopodia may play a role in NSCLC progression. The finding that some fraction or sub-type of NSCLC may use invadopodia as a mechanism of tumor growth and invasion may ultimately have clinical relevance. Our hypothesis is that invadopodia form in NSCLCs as a result of EGFr and KRas signaling and that this in turn promotes invasion and tumor cell growth. We will first evaluate the expression of invadopodia proteins in a panel of NSCLC lines previously phenotyped for EGFr and KRas status. Next we will determine the effect of invadopodia loss on invasion and tumor cell growth in vitro. Finally, we will evaluate the role of invadopodia in tumor growth and metastasis in vivo. This focused yet comprehensive analysis will elucidate the frequency of invadopodia involvement in the invasive behavior of lung cancer cells, and if appropriate, set the stage for more in depth studies.

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1. **INTRODUCTION:** Our hypothesis is that invadopodia form in NSCLCs as a result of EGFr and KRas signaling and that this in turn promotes invasion and tumor cell growth. We will test this hypothesis using a panel of NSCLC lines previously phenotyped for EGFr and KRas status. This focused yet comprehensive analysis will elucidate the frequency of invadopodia involvement in the invasive behavior of lung cancer cells, and if appropriate, set the stage for more in depth studies. These will likely involve the use of genetically engineered mouse models, as well as the evaluation of targeted inhibition of invadopodia formation.

2. **KEYWORDS:** Invadopodia, metastasis, invasion, KRas, EGF receptor

3. **OVERALL PROJECT SUMMARY:**

Months 0-3

Obtain and begin to evaluate 12 NSCLC cell lines for invadopodia formation, invasiveness and 3D growth.

8 cell lines were obtained and first evaluated for Tks5 expression. Then 7 of the 8 were tested for invadopodia formation and invasiveness (as judged by FITC gelatin degradation); the 8th was not suitable because it did not attach to the assay cover slips. Two of the cell lines (H1792 and H1650) were judged to be the best at forming invadopodia.

Infect NCI-H460 with lentiviruses expressing Tks5 or control (scrambled) shRNAs and puromycin select.

Prior to the initiation of the award, our preliminary analyses suggested that the H460 cells would in fact not be a good choice for the assays, and we therefore did not perform these experiments.

Months 4-6

Test control and Tks5 knockdown NCI-H460 cells in in vitro assays of invasion and 3D growth.

See above for why we chose not to work with this cell line.

Continue to evaluate 12 NSCLC cell lines for invadopodia formation, invasiveness and 3D growth.

The experiments described above (under months 0-3) were continued.

Infect other NSCLC cell lines which elaborate invadopodia with lentiviruses expressing Tks5 or control (scrambled) shRNAs and puromycin select.

Experiments were initiated to infect H1792 and H1650 cells with scrambled control and Tks5 specific shRNAs, with puromycin selection.

Months 7-9

Continue to infect other NSCLC cell lines which elaborate invadopodia with lentiviruses expressing Tks5 or control (scrambled) shRNAs and puromycin select.

The experiments described above were continued, such that the necessary cell lines were made.

Begin to test control and Tks5 knockdown NCI-H460 cells in orthotopic transplantation assays in nude mice.

As described above, the H460 cells were not suitable for our assays.

Test control and Tks5 knockdown NSCLC cell lines generated in months 4-6 in in vitro assays of invasion and 3D growth.

Experiments to test the engineered H1792 and H1650 cells were begun, and in vitro assays of invadopodia formation and invasion completed.

Months 10-12

Continue to test control and Tks5 knockdown NSCLC cell lines generated in months 7-9 in in vitro assays of invasion and 3D growth.

The 3D growth assays on the engineered H1792 and H1650 cells were completed.

Continue to test control and Tks5 knockdown NCI-H460 cells in orthotopic transplantation assays in nude mice.

As described above, the H460 cells were not suitable for our assays.

Test at least one other control and Tks5 knockdown NSCLC cell line in orthotopic transplantation assays in nude mice.

These studies have not yet started. They will be performed during the no-cost extension.

4. KEY RESEARCH ACCOMPLISHMENTS:

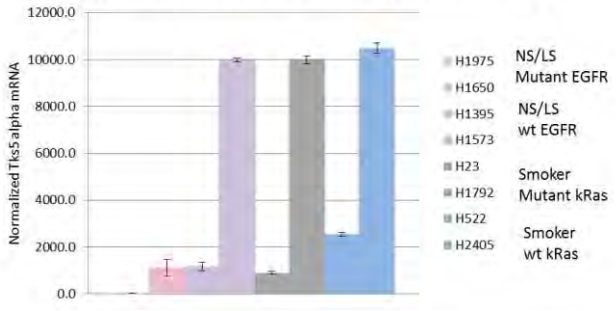
Major goals of the project

To evaluate NSCLC cell lines for invadopodia formation, invasiveness and 3D growth.

To use RNA interference to reduce Tks5 expression in NSCLC cell lines which elaborate invadopodia, and evaluate effect on invadopodia formation, invasiveness and 3D growth.

To test control and Tks5 knockdown NSCLC cells in transplantation assays in nude mice.

Fig 1. Analysis of Tks5 mRNA expression by qPCR



Accomplishments under these goals

In our original proposal, we discussed using H460 cells, since they are known to be invasive, and there was a possibility that they would form invadopodia. However, a preliminary assessment prior to beginning the award did not reveal robust invadopodia formation (not shown). We therefore decided to focus on other NSCLC cell lines. We obtained eight such lines: H1975 and H1650 (non-smoker, mutant EGFr); H1395 and H1573 (non-smoker, wildtype EGFr); H23 and H1792 (smoker, mutant Kras); and H522 and H2405 (smoker, wildtype KRas), and used qPCR to determine the levels of Tks5 mRNA (Figure 1). All expressed the mRNA, although the levels varied. Next, specific antibodies were used to determine the protein level of Tks5, and the related protein Tks4. NSCLC cells displayed variable levels of Tks5 and Tks4 proteins, with only H1395 expressing undetectable levels of Tks4. Neither the amount of Tks5 mRNA nor its protein level correlated with tumor site (primary or metastasis) or mutational status of KRas or EGF receptor in the lines analyzed.

Fig 2. Analysis of Tks protein levels

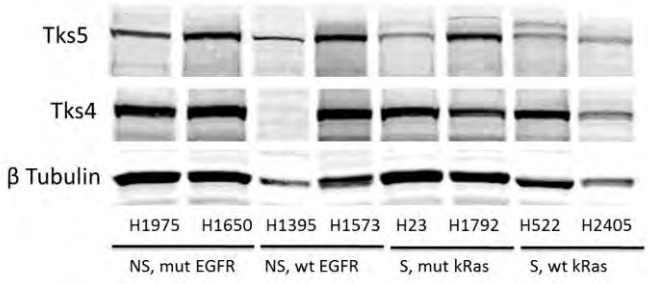
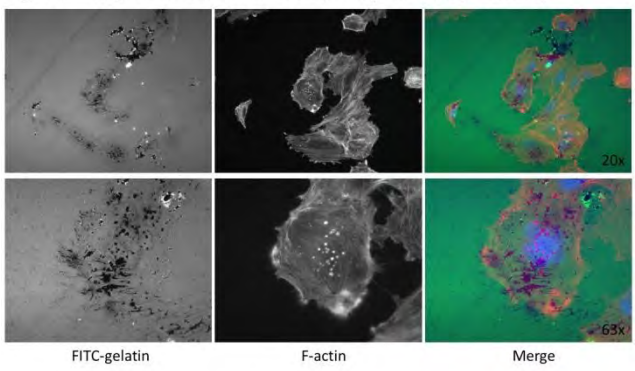


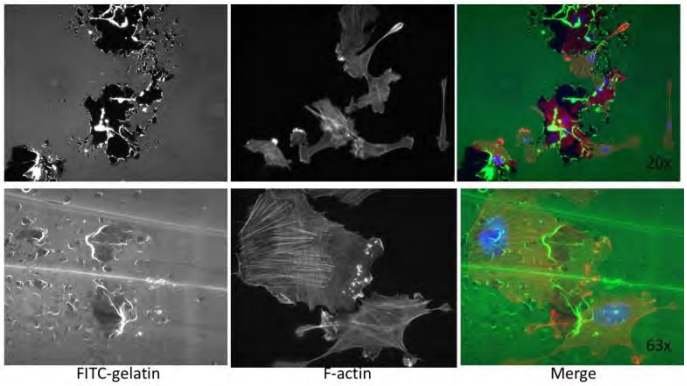
Figure 3. Invadopodia formation in H1792 cells (smoker, mutant KRas, metastasis)



Next, invadopodia formation was assessed. The ability of NSCLC lines to form invadopodia showed a partial correlation with the levels of Tks5. The lines H1792 (Figure 3) and H1650

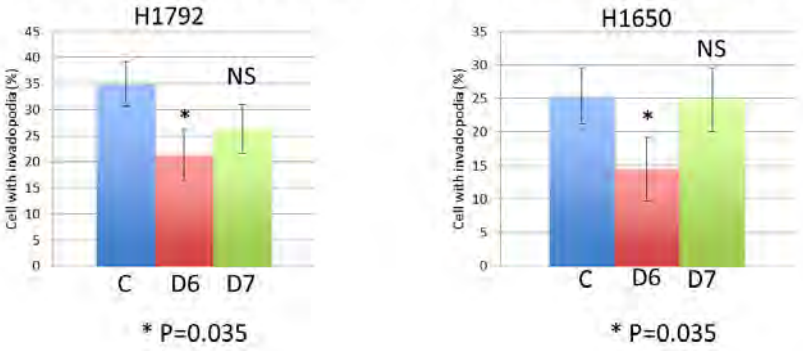
(Figure 4) efficiently elaborated invadopodia, and showed high and moderate Tks5 protein levels respectively. They were chosen for further analysis. Our preliminary data suggest that shRNA knockdown of Tks5 resulted in reduced invadopodia formation in these cells (Figure 5). However, only one of the 2 shRNAs used showed a strong effect, and these experiments will need to be repeated with fresh batches of lentiviral shRNA.

Figure 4. Invadopodia formation in H1650 cells (non-smoker, mutant EGFR, metastasis)



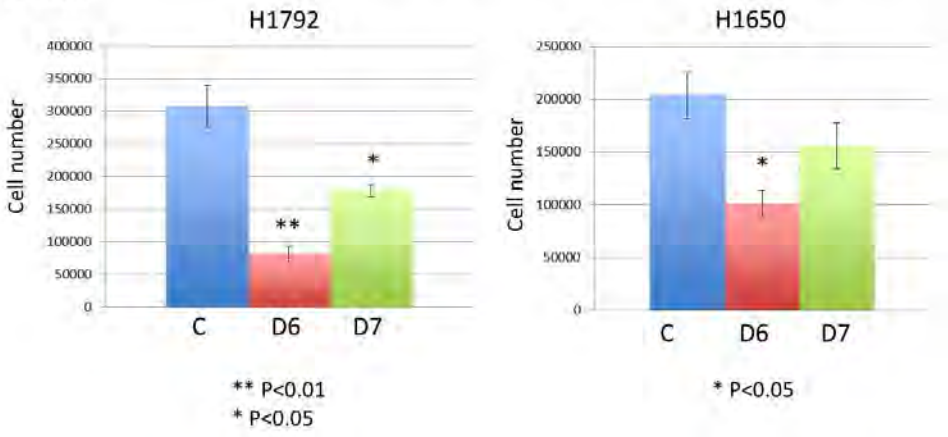
In other tumor types, we have unpublished data that invadopodia are required for tumor cells to grow when placed into extracellular matrix (3D assay). We conducted similar experiments with the H1792 and H1650 cells and determined that Tks5 is needed for 3D growth (Figure 6). These data provide strong support for testing whether Tks5 (and by implication invadopodia) are required for tumor growth in vivo. These experiments are in progress but not yet completed.

Figure 5. The effect of Tks5 knockdown on invadopodia formation



Since the submission of this proposal, a report was published (*Li CM*, *Chen G*, *Dayton TL*, *Kim-Kiselak C*, *Hoersch S*, *Whittaker CA*, *Bronson RT*, *Beer DG*, *Winslow MM*, *Jacks T*. *Differential Tks5 isoform expression contributes to metastatic invasion of lung*

Figure 6. The effect of Tks5 knockdown on the growth of cells in 3D collagen matrices (8 days)



adenocarcinoma. Genes & Dev. 2013. 27:1557-1567) that describes the importance of Tks5 in lung cancer invasion. This paper described the existence of two isoforms of the Tks5 mRNA (long and short) and concluded that the ratio of the two forms of Tks5 protein encoded by these mRNAs controlled invasive behavior. We too have determined that there are different isoforms of Tks5 mRNA (which we call α and β - *Cejudo-Martin P, Yuen A, Vlahovich N, Lock P, Courtneidge SA, Díaz B. Genetic disruption of the sh3pxd2a gene reveals an essential role in mouse development and the existence of a novel isoform of Tks5. PLoS One. 2014. 9:e107674*). However, we cannot confirm that the ratio of the two isoforms controls Tks5 biology. Indeed, we rarely detect the existence of the shorter protein isoform in cancer cells, including the lung cancer cell lines under study here. Nevertheless, it is possible that it is the ratio of the 2 mRNAs which is important. Therefore we plan to use specific primers to evaluate the expression of the short mRNAs of Tks5, and determine the ratios of short to long forms, in the NSCLC cells, and evaluate if there is any correlation with invadopodia formation, invasive behavior, 3D growth etc.

Opportunities for training and professional development
Nothing to report

Dissemination of results to communities of interest
Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?
We will use the no-cost extension period of this grant to repeat the in vitro and perform the in vivo analyses described above, and to prepare a manuscript describing our findings for publication.

5. **CONCLUSION:** We have determined that some NSCLC cell lines can form invadopodia, although no correlation was found between Ras or EGF mutational status and invadopodia competence. We further demonstrated that the invadopodia scaffold protein Tks5 appeared to be required for the growth of NSCLC cells in 3D. These data will be repeated for robustness, and then the effect of shRNA knockdown on tumor growth in vivo will be tested.

6. **PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:** NA

7. **INVENTIONS, PATENTS AND LICENSES:** NA

8. **REPORTABLE OUTCOMES:** NA

9. **OTHER ACHIEVEMENTS:** NA

10. **REFERENCES:** NA

11. **APPENDICES:** NA