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14. ABSTRACT Small cell lung cancer (SCLC) is the deadliest form of lung cancer and the most strongly associated with smoking history. Unfortunately, chemotherapy remains the main treatment option for patients with SCLC. Although this cancer typically responds extremely well, relapse is fast and largely inevitable. There are no effective therapies for relapsed tumors. The goal of our proposed study is to find new targets for drug therapy against relapsed SCLC tumors and provide continued hope for these patients. In this regard, we have identified a protein called HEPACAM2, which demonstrates unique, high expression in SCLC cell lines and tumors, making it a very attractive target. Knockdown of HEPACAM2 leads to cell cycle arrest, followed by apoptosis. Tankyrase 1 (TNKS) has been shown to poly(ADP-ribosylate) HEPACAM2 and this modification is necessary for HEPACAM2 spindle localization and mitosis. Specific inhibitors of TNKS have been developed with little activity towards PARP1, a participant in the DNA damage response.

We hypothesize that HEPACAM2 represents a novel therapeutic target in SCLC and propose two specific aims: (1) to determine the expression and cellular localization of HEPACAM2 in SCLC, and (2) to determine if SCLC cells demonstrate increased sensitivity to TNKS inhibitors. HEPACAM2 expression will be tested at the mRNA and protein levels by qPCR and western blotting, respectively. Subcellular localization will be determined by confocal microscopy. Expression will be determined in multiple lung cancer cell lines, both SCLC and NSCLC, along with non-lung and normal cell lines as additional controls. Tumors will be investigated for expression, which will be correlated with survival. Inhibition of HEPACAM2 function will be tested by siRNA knockdown and small molecule inhibitors of TNKS using cell proliferation, migration and apoptosis assays. Synergism with other PARsylation inhibitors will also be explored.

This proposal will address the Area of Emphasis '*to understand predictive and prognostic markers to identify responders and non-responders'. HEPACAM2* expression could represent a predictive marker in this cancer showing sensitivity to TNKS inhibitors. In addition, its potential for variable expression in SCLC tumors could make its expression prognostic for survival or chemo-response.

15. SUBJECT TERMS
Small cell, lung cancer, HEPACAM2, poly-ADP-ribosylation, PARP, tankyrase

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1. Introduction:

The goal of this study was to find new targets for drug therapy against relapsed small cell lung cancer (SCLC) tumors. In this regard, we focused on a protein called HEPACAM2, which appeared to be highly and specifically expressed only by SCLC tumors, making it a very attractive target. We sought to explore if HEPACAM2 was important for SCLC growth and, if so, how best to target it. Therefore, the aims of the study were to first determine the expression and cellular localization of HEPACAM2 in SCLC. Subcellular localization would be determined by confocal microscopy. Expression would be determined in multiple lung cancer cell lines, both SCLC and NSCLC, along with non-lung and normal cell lines as additional controls. Tumors would be investigated for expression, which would be correlated with survival. The second aim was to determine if HEPACAM2 could be used to specifically target SCLC cells using TNKS inhibitors. Tankyrase 1 (TNKS) has been shown to poly(ADP-ribosylate) HEPACAM2 and this modification is necessary for HEPACAM2 mitotic spindle localization and mitosis. Specific inhibitors of TNKS have been developed with little activity towards PARP1. Thus, SCLC may demonstrate particular sensitivity to TNKS inhibitors, leading to alterations in HEPACAM2 function measured by cell proliferation and apoptosis assays. Synergism of TNKS inhibitors with other PARsylation inhibitors would also be explored

We requested and received a no-cost extension for a year to allow us to continue pursuing these aims and to overcome some unexpected problems. This represents our final report and builds on what was previously reported in our 2018 annual report. As such, it incorporates data/text from the annual report and adds new data we gathered during the extension.

2. Keywords:

Small cell lung cancer, SCLC, HEPACAM2, HEPACAM, poly-ADP-ribosylation, PARP, tankyrase, TNKS, PARsylation

3. Accomplishments:

- **What were the major goals of the project? What was accomplished under these goals?** The goals are listed following the *revised* Specific Aims/tasks of the SOW (see Appendix) and are followed by relevant accomplishments.

Specific Aim 1: To determine the expression and cellular localization of HEPACAM2 in SCLC:

Major Task 1: To explore HEPACAM2 expression in SCLC and control cell lines (*ALL commercially available*: **SCLC** H1694, H211, H1092, H1048, SHP77, H2141, H209, H82, DMS114, SW1271, H841, H1184; **NSCLC** A549, SW900, H2009, H1299, H520; **NORMAL** NL20):

Subtask 1: Perform Taqman PCR.

Our initial excitement to study HEPACAM2 was based on data we extracted from the Cancer Cell Line Encyclopedia (CCLE) database (https://portals.broadinstitute.org/ccle/page?gene=RUNX1T1) demonstrating that *HEPACAM2* expression was highly enriched in SCLC cell lines compared to NSCLC, as well as to all other cancer cell lines in the CCLE. Thus we began our study by using qPCR (Taqman) to validate the mRNA expression levels of both *HEPACAM2* and *HEPACAM* among an assortment of SCLC, NSCLC, mesothelioma and normal lung cell lines. The results, shown on the left in Table 1, show that SCLC cell lines (red text) clearly express much higher levels of *HEPACAM2* compared to NSCLC (blue text), mesothelioma (yellow text) and normal lung (black text) cell lines, and that the rank order of expression generally parallels that found by the CCLE dataset. We then performed qPCR for *HEPACAM* on the same samples and found, similar to the CCLE database, that *HEPACAM* mRNA was undetectable in SCLC and also very low in other thoracic cancer cells (right side of Table 1). It should be noted that CCLE values are listed as log2, meaning that every 1.0 point change in value = two-fold change in mRNA expression. Our qPCR results are reported as the ratio *HEPACAM2* mRNA normalized to that of β-actin. Taken together, these results confirm the highly specific expression of *HEPACAM2*

in SCLC and establish conditions to validate antibody results based upon the large difference in expression of *HEPACAM2* between SCLC vs NSCLC.

Table 1: Validation of *HEAPACAM2* mRNA expression by qPCR.

Hepacam2 q-PCR Basal Levels		Hepacam2 CCLE mRNA Levels			Hepacam q-PCR Basal Levels		Hepacam CCLE mRNA Levels	
H1694	4.318	H1694	13.5	SCLC	H520	2.15E-04	A549	4.73
DMS454	1.537	DMS454	12.61	NSCLC	NL-20	2.29E-05	H1869	4.68
H211	0.3383	H211	12.37	Meso	H2052	8.63E-06	H2029	4.62
H1092	0.2252	H69	12.36	Normal	Calu I	1.89E-06	H1299	4.61
H69	0.1761	H1092	12.01		H1694		H520	4.47
H2029	0.1574	H2171	11.34		DMS454		H211	4.36
H2171	0.115	H2029	11.16		H211		H209	4.34
H1048	0.0875	H1048	10.37		H69		SHP77	4.32
H526	0.0642	H526	9.91		H1092		Calu I	4.33
H209	0.0482	H209	8.44		H2171		H1975	4.32
SHP77	0.0095	H2141	7.77		H2029		DMS454	4.28
H2141	0.0057	SHP77	7.49		H1048		H1694	4.26
H1299	0.000166	H1869	3.78		H209		H2141	4.24
H520	0.000038	H1975	3.74		H2141		H1048	4.23
Calu I	5.17E-06	A549	3.72		H526		H2171	4.22
H1975	4.17E-06	H520	3.72		SHP77		H69	4.2
H2052	4.16E-06	H1299	3.69		H1299		H1092	4.2
NL-20	2.18E-06	Calu I	3.66		A549		H2052	4.15
A549	2.15E-06	H2052	3.58		H1869		H526	3.97
H1869	1.77E-06	NL-20	N/A		H1975		NL-20	N/A

Next, we wanted to obtain evidence that *HEPACAM2* is highly and specifically expressed only in SCLC tumors. Thus, we compared RNAseq data of SCLC tumors (Rudin et al, 2012 dataset PMID 22941189) to that of others cancers found in the TCGA database, and again found that *HEPACAM2* mRNA is highly enriched in SCLC tumors (see Figure 1), validating the results we obtained above for SCLC cell lines.

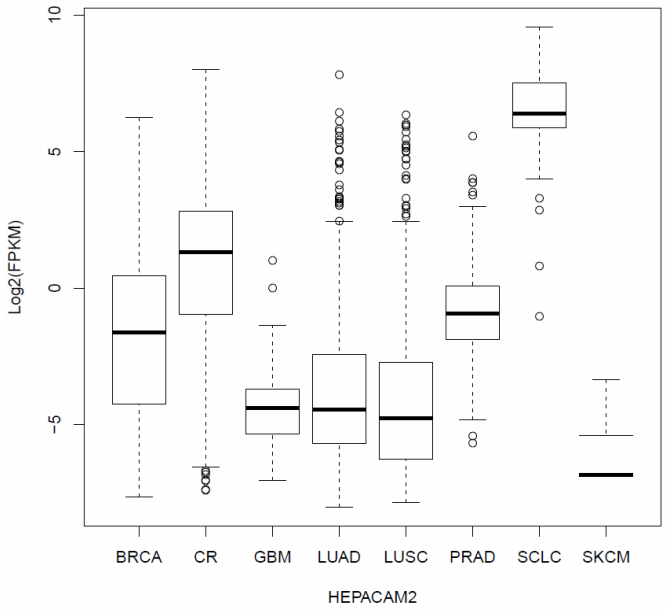

Figure 1: Boxplot of *HEPACAM2* mRNA expression in tumors. Determined from RNAseq data. BRCA: breast cancer, CR: colorectal cancer, GBM: glioblastoma, LUAD: lung adenoma NSCLC, LUSC: lung squamous NSCLC, PRAD: prostate adenoma cancer, SKCM: skin melanoma cancer.

In addition, to provide more controls for our HEPACAM2 protein expression experiments, we also decided to over-express and/or knockout *HEPACAM2* in lung cancer cells having various endogenous *HEPACAM2* mRNA levels (see Table 1). Initially we ordered a C-terminal, FLAG-tagged *HEPACAM2* lentivirus from Genscript. The reason for placing the FLAG-tag at the C-terminal was because HEPACAM2 has a predicted N-terminal signal sequence that may be disrupted by the FLAG tag or make the tag susceptible to removal during post-translational processing (UniProt). We have used this to produce stable over-expression (OE) of *HEPACAM2* in 10 cell lines: H1299, H1869, A549 and H2009 (all NSCLC) and H841, SW1271, SHP77, H2171, H1694 and H211 (all SCLC). Interestingly, using qPCR, we could only obtain over-expression of *HEPACAM2* mRNA in cells with low endogenous mRNA levels (see Figure 2). This was true for both NSCLC (H2009, A549, H1869, H1299) and SCLC (H841, SW1271, SHP77) cells. SCLC cells with

high endogenous HEPACAM2 mRNA (H2171, H211, H1694) could not be induced to express higher levels. We are unsure why this occurs, but it remains an interest for future studies.

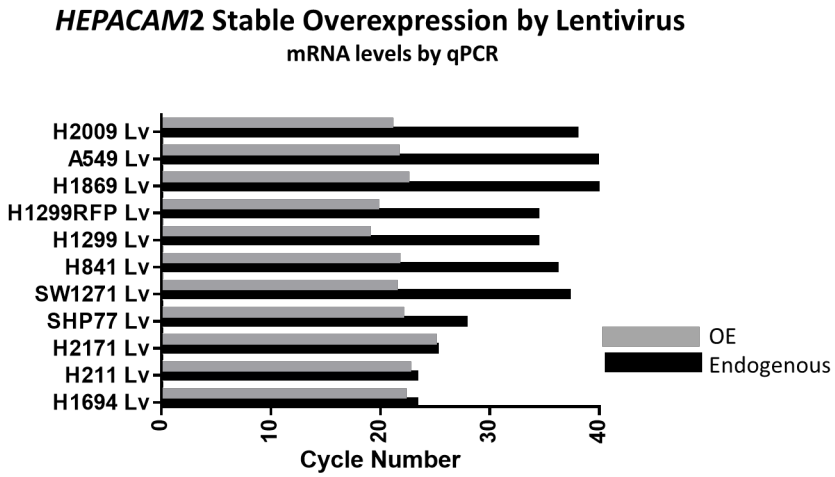

Figure 2: Over-expression of *HEPACAM2* mRNA in lung cancer cells. qPCR was used to measure mRNA in both parental cells (black bars) and in cells stably over-expressing HEPACAM2 (grey bars). The x-axis represents the C_P values; thus the higher the value the lower the amount of PCR amplicon detected. A value of '40' represents undetectable amplicon levels. H1299RFP is just H1299 stably transfected with an RFP construct, which should not affect *HEPACAM2* expression, as observed.

We also used CRISPR to stably knockout (KO) *HEPACAM2* mRNA in four SCLC cell lines with high endogenous *HEPACAM2* expression: DMS454, H1694, H2029 and H69 cells. Three different guide RNAs were used, which means we established 12 new stable cell lines with putative *HEPACAM2* knockout. The CRISPR guides were obtained from Genscript in lentiviral backbones, which we then packaged into viral particles and infected into each cell line. When we performed qPCR for *HEPACAM2* mRNA measurement in the CRISPR pools; little or no detectable KO was observed. We were confident the cells were expressing the construct, however, because of their resistance to the selection marker, puromycin. Apparently, qPCR is occasionally unable to detect CRISPR KO because of the nature of the gene splicing event, so we assumed it worked in our cells based on their puromycin resistance.

Finally, we also tried to transiently knockdown (KD) *HEPACAM2* using Dharmacon siRNA Smartpools. The assay to detect KD was qPCR. Again, we failed to detect KD after several attempts, using DMS454 cells that show high endogenous *HEPACAM2* expression.

Taken together, our data shows that HEPACAM2 is specifically expressed in SCLC and we developed both HEPACAM2 OE and KO models to investigate its detection and role in SCLC.

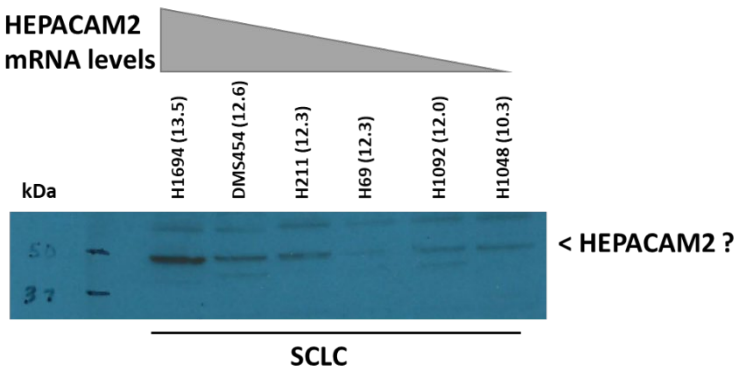

Figure 3: HEPACAM2 expression by western blotting: Western blotting results of SCLC cell line protein lysates using Bioss (#8417R) antibody. Cells are arranged, left to right, from highest to lowest *HEPACAM2* mRNA levels. Arrow indicates putative HEPACAM2 band, which is reported to have a molecular weight of 52 kDa. The numbers in parentheses indicate mRNA expression values from the CCLE.

Subtask 2: Perform western blots on protein lysates.

We initially tried to find a multi-purpose antibody that we could use for western blotting, immuno-histochemistry (IHC) and confocal imaging, as these were major goals outlined for Aim 1. We chose an antibody from Bioss listed with all these features. We initially tested it by western blotting using SCLC cell lines having high levels of *HEPACAM2* mRNA and obtained a relatively clean band of about the expected size for HEPACAM2 = 52 kDa whose pattern of expression followed that of *HEPACAM2* mRNA levels (see Figure 3). Encouraged by these results we began parallel experiments to use this antibody for immuno-fluorescent (IF) studies of

cells and IHC of tumors. The results were disappointing. For immuno-fluorescent (IF) staining of single cells, we stained DMS454 SCLC cells having high HEPACAM2 mRNA levels and NSCLC A549 cells as a negative control and obtained the same strong staining throughout the cells; no specific subcellular localization or SCLC specificity could be discerned despite multiple permutations to the staining protocol. In parallel we obtained IHC results of a TMA containing tissue cores of 22 SCLC and 12 NSCLC tumors, where again no specific staining of SCLC was observed relative to NSCLC. Thus, we abandoned further use of the Bioss antibody.

We next tried an antibody from Novus that was approved for western blotting. We hoped that it could also be useful for future IF and IHC experiments. When we western blotted an extended panel of cell lines with this antibody, a band of lower than expected size of HEPACAM2 was again observed whose expression roughly paralleled that of *HEPACAM2* mRNA levels (see Figure 4). Although this lower band was similar in size to that visualized by the Bioss antibody, the results did not match (see H69), giving us some concern, as did the stronger band of higher molecular weight that did not correlate with *HEPACAM2* mRNA expression (labeled NS in Figure 4).

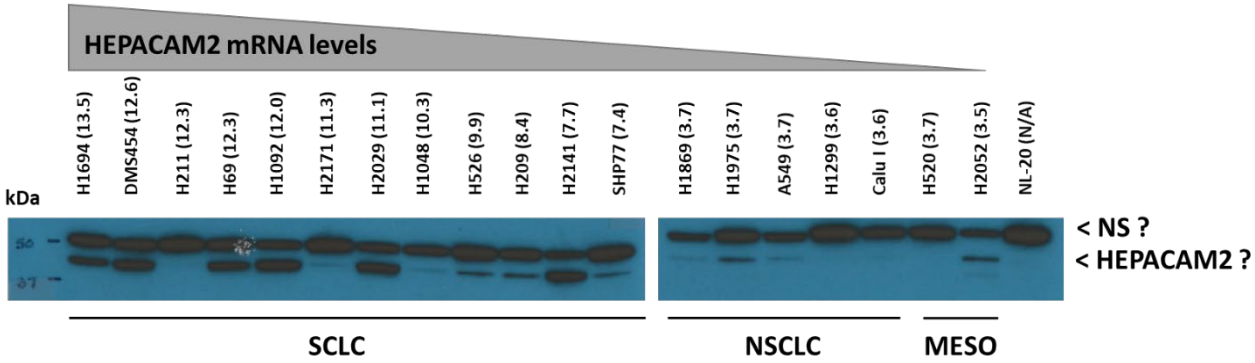

Figure 4: HEPACAM2 expression by western blotting: Western blotting results of different thoracic cell protein lysates using Novus (#NBP1) antibody. Cells are arranged, left to right, from highest to lowest *HEPACAM2* mRNA levels. Arrow indicates putative HEPACAM2 band, as well as a putative non-specific band (NS). MESO = mesothelioma. NL20 is a 'normal' lung cell line. The numbers in parentheses indicate mRNA expression values from the CCLE.

Because of our uncertainty detecting endogenous HEPACAM2 protein in western blots, we purchased two new antibodies from Aviva (epitopes in N-terminal and mid regions of protein) and one from ProSci (epitope in C-terminal region). The Novus antibody (Figure 4) recognizes the exact same mid region of HEPACAM2 as Aviva. Thus, we had antibodies directed at many different epitopes of the HEPACAM2 protein. We hoped that the use of multiple antibodies, when coupled with the stable HEPACAM2 OE and KO cells to serve as positive and negative controls, respectively, would successfully identify the true HEPACAM2 band in western blots.

Over-expression of HEPACAM2 was detected as a new ~70 kDa band using anti-FLAG antibody in H1299 cells (see Figure 5, left). This band could also be observed with the ProSci antibody (Figure 5, right), but not with either of the Aviva antibodies nor the Novus antibody (data not shown). When the cells were grown in the presence of tunicamycin, which blocks N-terminal glycosylation of membrane proteins, this led to the appearance of a ~50 kDa band in H1299 and H1869 cells (Figure 5), similar to the predicted size for HEPACAM2. We performed the tunicamycin treatment because we found an analysis of the amino acid sequence of HEPACAM2 that predicted a single-pass membrane protein with many potential N-linked glycosylation sites. Parallel analysis of two SCLC lysates with high endogenous *HEPACAM2* expression (H1694 and H526) did not yield any similar ~70 or ~50 kDa bands (Figure 5, right). From these results we concluded: 1) endogenous HEPACAM2 is not detectable by simple western blotting of cell lysates using any commercial antibody, 2) over-expressed (OE) HEPACAM2 can be detected in some (H1299 is best), but not all, transfected cells lines for reasons that are unclear, 3) OE HEPACAM2 is glycosylated, suggesting a plasma membrane localization, and 4) tunicamycin may be a useful tool to concentrate and better visualize OE HEPACAM2 detection.

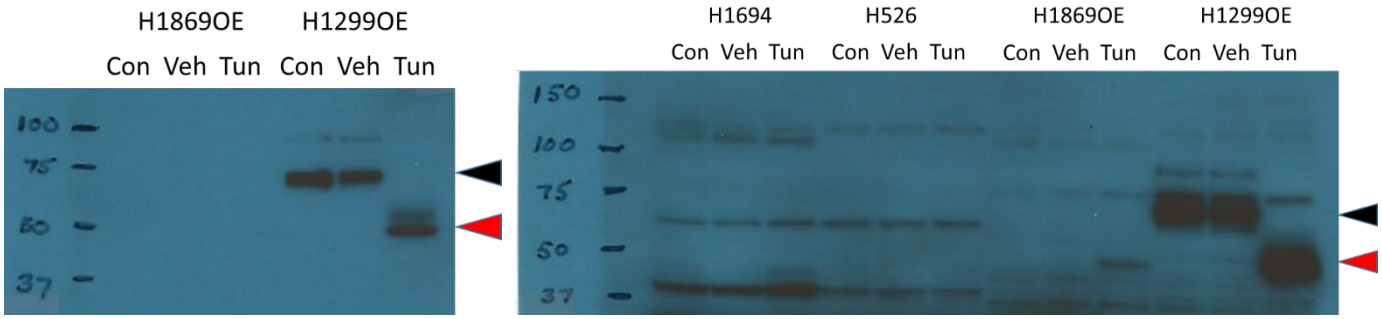

Figure 5: HEPACAM2 OE detection by western blotting: Western blotting results for protein lysates using FLAG (left) and ProSci C-terminal antibody (right). Two cells (H1694 and H526) are untransfected but have high endogenous *HEPACAM2* mRNA, and two cells (H1869 and H1299) stably over-express HEPACAM2. Black arrow indicates putative ~70 kDa intact HEPACAM2 band in OE cells, while red arrow indicates putative non-glycosylated HEPACAM2 band of ~50 kDa in OE cells. Con: control, Veh: vehicle treated, Tun: tunicamycin (1 µg/ml) treated 24 h. MW markers on left.

Next, we used immuno-precipitation (IP) as a potential tool to increase detection of both endogenous and OE HEPACAM2. In OE cells, we IP'd 500 µg lysate with anti-FLAG (mouse) antibody and used the ProSci (rabbit) antibody for detection. As shown in Figure 6, this procedure made detection of OE HEPACAM2 easier in most cells. Two interesting observations came out of this experiment. First, a second band at ~90 kDa was detected that was only weakly observed in the input lysates. Second, re-probing the blot with the Aviva N-terminal antibody could also detect the same two bands in H1299 cells, although with much less intensity (data not shown). This result was important because it indicated expression of the full-length protein, as both the FLAG-tag and ProSci antibodies detect the C-terminus of HEPACAM2. The Aviva mid-region antibody, however, did not work.

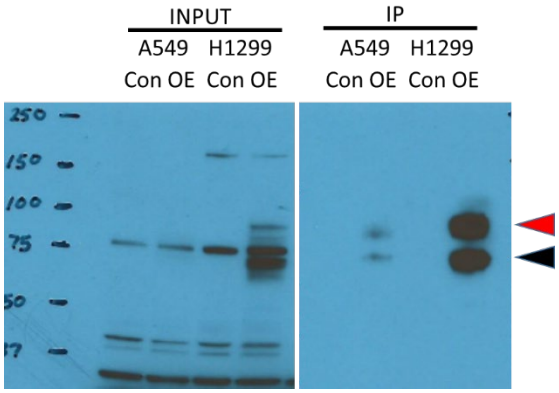

Figure 6: IP of OE HEPACAM2: Western blotting results for protein lysates following FLAG IP and western blotting with ProSci antibody. Results from two cell lines (A549, H1299) that were either untransfected or stably over-expressing HEPACAM2 are shown. Black arrow indicates putative ~70 kDa intact HEPACAM2 band previously observed in OE cell lysates, while red arrow indicates new HEPACAM2 band of ~90 kDa enriched after IP in OE cells. Con: untransfected cells, OE: HEPACAM2 over-expressing cells. MW markers on left. 50 µg of input lysate were loaded onto gels and the equivalent of 500 µg lysate was used for IPs.

Because IP increased our detection of OE HEPACAM2, we attempted to modify the protocol to detect endogenous HEPACAM2. This required using the ProSci antibody for both the IP and detection of HEPACAM2. This should work as long as we cross-linked the antibody to the agarose beads before the initial pull-down to avoid massive IgG detection during the western blot procedure. As shown in Figure 7. This modified IP protocol was able to detect OE HEPACAM2 in some cell lines (SW1271, A549, H1299), but not all.

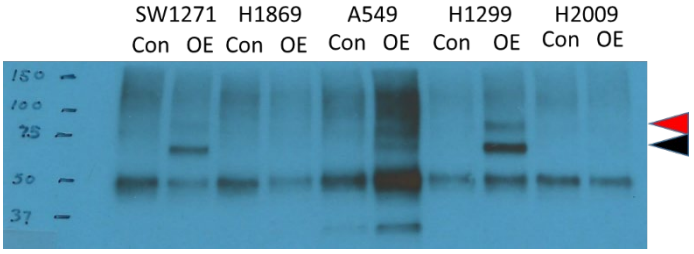

Figure 7: IP of OE HEPACAM2: Western blotting results for protein lysates following IP and western blotting with ProSci antibody. Results from five cell lines that were either untransfected or stably over-expressing HEPACAM2. Black arrow indicates putative ~70 kDa intact HEPACAM2 band, while red arrow indicates new HEPACAM2 band of ~90 kDa enriched after IP in OE cells. Con: untransfected cells, OE: HEPACAM2 over-expressing cells. MW markers on left. 50 µg of input lysate were loaded onto gels and the equivalent of 500 µg lysate was used for IPs. The ~50 kDa band is likely detection of non-crosslinked IgG from beads.

Unfortunately, the modified IP protocol was unable to detect endogenous HEPACAM2 in several cell lines with high *HEPACAM2* mRNA levels (data not shown).

Lastly, because HEPACAM2 is predicted to be a cell surface protein (http://wlab.ethz.ch/surfaceome/), we used a cell-surface biotin-labelling technique as an alternative method to pull-down HEPACAM2 from cell lysates and thereby improve our ability to detect endogenous HEPACAM2. Briefly, cell surface proteins are biotinylated on intact cells, cell lysates are made, then biotinylated proteins are pulled-down from protein lysates with strep-avidin beads and the ProSci antibody is used for western blotting. We used this method on parental and HEPACAM2 OE H1299 cells, as well as on parental and CRISPR-treated H1694 cells. The results shown in Figure 8 demonstrate that this technique worked well for H1299 cells which OE HEPACAM2, but did not work for H1694 cells with high endogenous HEPACAM2 expression. The broad band detected at ~150 kDa likely represents a fully glycosylated form of HEPACAM2.

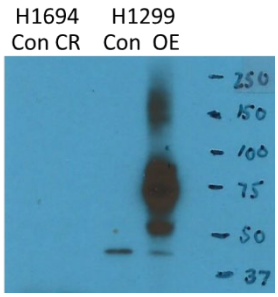

Figure 8: Biotinylation of HEPACAM2: Western blotting results for protein lysates following cell surface biotinylation and strep-avidin pull-down. Western blotting used the ProSci antibody. Results from two cell lines (H1694, H1299) that were either untransfected (Con), CRISPR-treated (CR) or stably over-expressing HEPACAM2 (OE) are shown. MW markers on right. The equivalent of 500 µg lysate was used for pull-downs.

What can we conclude from all of these experiments? First, we are unable to detect endogenous HEPACAM2 despite high mRNA levels. Second, we can detect OE HEPACAM2 in H1299 cells, and to a lesser extent in other OE cell lines. Three, OE HEPACAM2 is an N-glycosylated protein sensitive to tunicamycin treatment and is localized to the cell surface. It is unclear what percentage of OE HEPACAM2 is localized to the cell surface.

Subtask 3: Perform confocal microscopy:

See Major Task 1, subtask 2 immediately above. We did try with the Bioss antibody but there was no difference in staining intensity or location between SCLC (DMS454) and a NSCLC (A549) cells, contrary to our mRNA expression analysis of SCLC cell lines. Our data in Figure 8, however, strongly suggest that at least OE HEPACAM2 is located on the cell surface in H1299 cells.

Major Task 2: To analyze HEPACAM2 expression in SCLC tumors:

Subtask 1: Optimize IHC detection of HEPACAM2 in SCLC tumors.

See Major Task 1, subtask 2 immediately above. We did try with the Bioss antibody on a mixed lung cancer TMA but there was no difference in staining intensity between SCLC and NSCLC, contrary to our mRNA expression analysis of SCLC cell lines and tumors. Thus, we judged this IHC to be unreliable. Although we did purchase three additional antibodies to detect HEPACAM2, we were unable to validate their specificity in western blotting experiments (see above) and so did not want to use them on TMAs, which are a valuable and limited resource.

Subtask 2: IHC stain 2-3 SCLC TMAs for HEPACAM2.

See Major Task 2, subtask 1 immediately above.

Subtask 3: Read & correlate IHC staining with clinical outcomes.

See Major Task 2, subtask 1 immediately above.

Specific Aim 2: To determine if SCLC cells demonstrate increased sensitivity to TNKS inhibitors and the phenotype of HEPACAM2 OE/KD cells :

Major Task 1: Determine efficacy of TNKS inhibitors in SCLC and NSCLC cells as controls: (subset of cells named above).

Subtask 1: Determine efficacy on cell growth, adhesion & migration using IncuCyte ZOOM.

Independent of our experiments for Specific Aim 1 we began to look for effects of TNKS inhibitors on SCLC cell growth. Initially we choose the drug NVP-TNK S656 because of its high potency (IC50 6 nM) and selectivity (300-fold over PARP1/2). We choose three cell lines each with high (H1694, H2171, H1048) versus low (H446, SW1271, DMS114) HEPACAM2 mRNA levels. We proposed that SCLC cells with high HEPACAM2 expression would be most sensitive to growth inhibition by TNKS inhibitors.

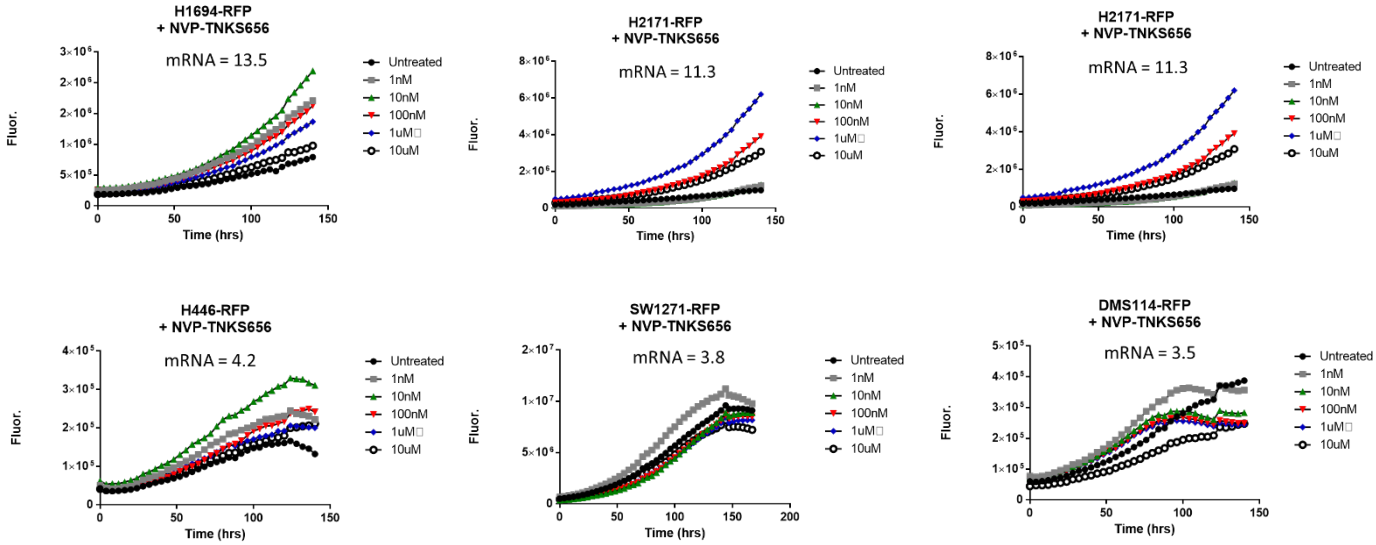

Figure 9: Effects of drug TNKS656 on SCLC cell growth: Real time measurement of RFP-labeled cell growth over 150-200 hr in the presence of five concentrations of drug (0–10 μM) using IncuCyte ZOOM. Top three cells express high and bottom three cells express low HEPACAM2 mRNA levels. Numbers for mRNA expression values from the CCLE.

Our results, shown in Figure 9, demonstrated that at a dose of 100 nM (red line), cells with high *HEPACAM2* mRNA exhibited *increased* cell growth, whereas growth in cells with low HEPACAM2 expression were relatively unaffected compared to untreated cells (black line). This result is opposite to what we expected and ruled out continuing this task any further.

Subtask 2: Determine effects on subcellular localization.

See Specific Aim 1, Major Task 1, subtask 3 above for our experimental conclusions on HEPACAM2 subcellular localization. Because we found that HEPACAM2 is likely localized to the cell surface and not the mitotic spindle, as reported in the literature, the whole mechanism of action of TNKS on HEPACAM2 function was brought into question. Thus, we discontinued this line of experiments.

Major Task 2: Determine effect of HEPACAM2 over-expression and knockdown: (subset of cells named above).

Subtask 1: Determine effects on cell growth, adhesion & migration using IncuCyte ZOOM .

We initially noticed that adherent H1299 cells with OE HEPACAM2 are *very* difficult to trypsinize off the dish when we needed to split them. This acquired phenotype might make sense if HEPACAM2 is a cell surface protein, as our data above suggest, and not a mitotic spindle protein. Thus, we decided to explore the effects of HEPACAM2 over-expression and knockdown on cell migration using a wound healing, or scratch assay. Once again we used the IncuCyte ZOOM for real time measurement of RFP-labeled cell behavior on scratch wound assay plates the company provides. Initial results revealed that both H1299 and A549 cells with HEPACAM2 OE demonstrated slower scratch wound healing compared with parental controls (Figure 10, left and center). This behavior was not due to any measurable difference in cell proliferation (Figure 10, right).

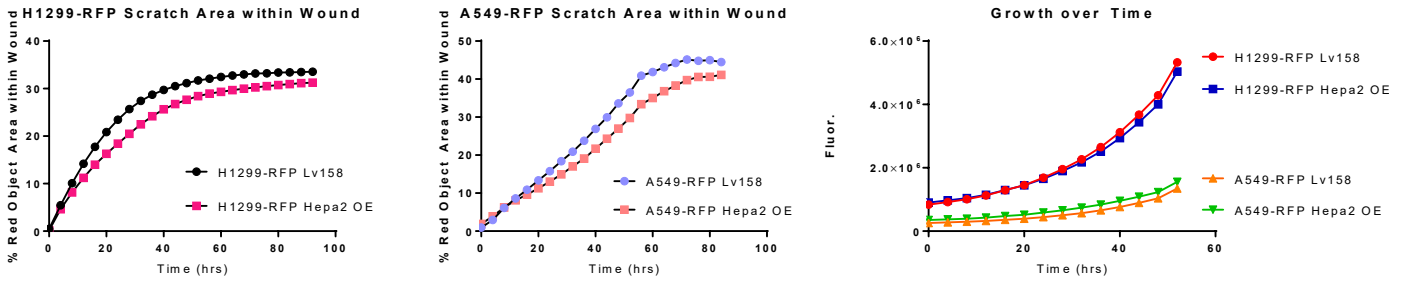

Figure 10: Effect of HEPACAM2 OE on wound healing and growth: Real time measurement of RFP-labeled cell behavior over 60-100 hr using the IncuCyte ZOOM. The left and center panels show results of scratch wound assays, while the panel at right shows proliferation curves. LV158 is an empty vector control for HEPACAM2 OE vectors.

We then performed similar experiments using cells with stable CRISPR integration to KO HEPACAM2 expression. If anything, HEPACAM2 KO decreased wound healing in scratch wound assays (Figure 11) but slightly increased cell proliferation (Figure 12). These experiments on CRISPR cells are hard to interpret, however, because we do not know with certainty if the CRISPR decreased HEPACAM2 levels, or by how much, at an mRNA or protein level (see Specific Aim1, above).

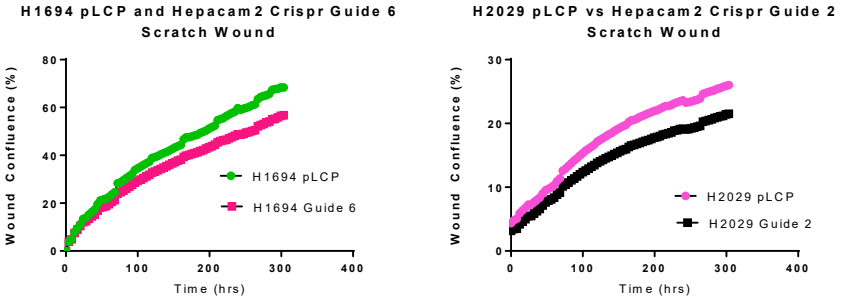

Figure 11: Effect of HEPACAM2 KO on wound healing: Real time measurement of RFP-labeled wound repair over 300 hr using the IncuCyte ZOOM. pLCP is an empty vector control for HEPACAM2 KO CRISPR guide vectors.

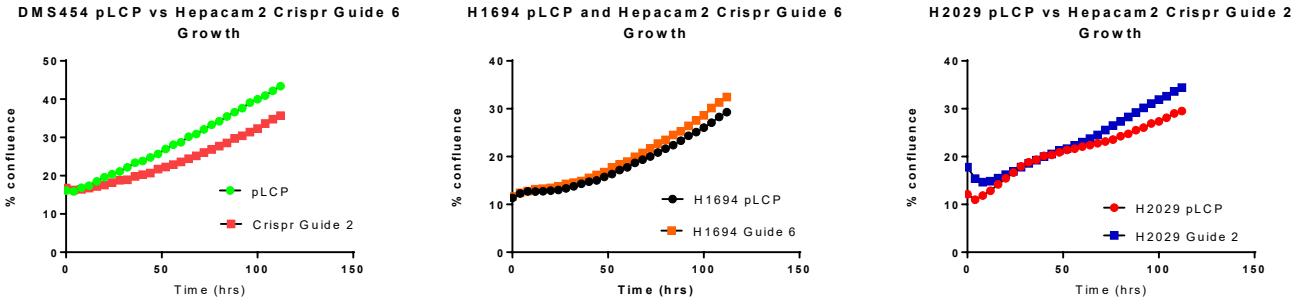

Figure 12: Effect of HEPACAM2 KO on cell growth: Real time measurement of RFP-labeled cell growth over 150 hr using the IncuCyte ZOOM. pLCP is an empty vector control for HEPACAM2 CRISPR vectors.

Subtask 2: Determine effects on subcellular localization.

See results of Specific Aim 1, Major Task 1, subtask 3 above.

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

Nothing to report.

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

Although none of this work has been presented at any conference or published, we intend to follow up the question of HEPACAM2 OE increasing cell adhesion and migration as a novel phenotype that may have some application to cancer metastasis.

- **What do you plan to do during the next reporting period to accomplish the goals?**

N/A

4. Impact:

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

Taken together, we have:
1) confirmed the specific expression of *HEPACAM2* mRNA in SCLC, but not in NSCLC or other cancers
2) shown that OE HEPACAM2 is a cell surface protein which is modified by N-terminal glycosylation
3) indicated a potential role for HEPACAM2 in cell migration/metastasis

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

Our results indicate that HEPACAM2 may provide the basis for two therapeutic strategies in SCLC:
1) by development of an antibody-drug complex carrying a lethal payload using an antibody that recognizes extracellular HEPACAM2 epitopes
2) by using HEPACAM2 as a SCLC-specific target for CAR T-cell therapy directed at binding partners of HEPACAM2

- **What was the impact on technology transfer?**

Nothing to report.

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

Nothing to report.

5. Changes/problems:

- **Changes in approach and reasons for change.**

We changed our original SOW by adding the establishment of HEPACAM2 OE and CRISPR KO cells to provide controls for our protein detection methods. Unfortunately, these efforts did not help us to identify any antibodies that would be useful for detection of endogenous HEPACAM2 protein in confocal and TMA experiments. These new cells did create, however, a new experimental direction to study the role of HEPACAM2 in cell adhesion and metastasis. Finally, we discontinued our pursuit of TNKS inhibitors as potential therapeutic agents in SCLC

because 1) they actually increased cell growth, and 2) our data was not consistent with a mitotic spindle location for HEPACAM2, which was necessary for TNKS activity.

- **Actual or anticipated problems or delays and actions or plans to resolve them.**

Our primary difficulty was in detecting endogenous HEPACAM2 protein expression. We devoted a majority of our efforts into solving this problem (see Specific Aim1, subtask 2) but could not resolve the issue. This blocked our ability to perform several proposed tasks.

- **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. Participation & other collaborating organizations:

- **What individuals have worked on the project?**

Name:	Afshin Dowlati, MD
Project role:	PI
Researcher identifier:	Orchid # 0000-0003-4535-6839
Nearest person months worked:	24
Contribution to project:	PI- oversight and direction.
Funding support:	This and grants listed below and University Hospitals of Cleveland research support.

Name:	Karen McColl, BS
Project role:	Lab manager
Researcher identifier:	N/A
Nearest person months worked:	24
Contribution to project:	Ms. McColl has worked on all aspects of this project.
Funding support:	This and University Hospitals of Cleveland research support.

Name:	Gary Wildey, PhD
Project role:	Program manager
Researcher identifier:	Orchid # 0000-0001-7105-1313

Nearest person months worked:	24
Contribution to project:	Research advise and report writing.
Funding support:	University Hospitals of Cleveland research support and grants listed below.

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

Yes, one new grant was funded, one was discontinued:

Active:

U24 (Rudin, PI) 02/20/17-01/31/22 0.6 calendar
NIH/NCI $75,000 (sub only)
Small-cell Lung Cancer (SCLC) Consortium: Coordinating Center
Subproject: *Construction of SCLC tissue micro-arrays*

Here we propose to construct multiple tissue micro-arrays from de-identified small-cell lung cancer tumor specimens and provide matching genomic profiling data on tumor specimens with sufficient tissue. Additional data on patient and tumor features will be provided for each tumor specimen placed in the TMA.
Role: Leader, tissue micro-array subproject.
Overlap: None- no funds are requested to make the TMAs used in the current application, as stated in the Budget Justification.

R21 (Dowlati, PI) 07/01/18-06/30/20 1.2 calendar
NIH/NCI $275,000
Identification and targeting of chemotherapy refractory small cell lung cancer

Here we propose to determine whether *RB1* mutation status and/or protein expression identifies chemo-refractory patients in small-cell lung cancer (SCLC). A secondary aim is to determine whether *RB1* mutation status and/or protein expression predicts sensitivity to CDK4/6 inhibitors in SCLC models.
Overlap: None.

Discontinued:

Concept Award (Dowlati, PI) 09/01/16-08/31/18 1.2 calendar
DOD < $60,000 (no cost extension)
RUNX1T1 amplification induces 'small cell' cancer

Here we examine the role of RUNX1T1 in small-cell lung cancer and the potential role it may play in non-small to small-cell lung cancer transformation.
Overlap: None.

- **What other organizations were involved as partners?**

Nothing to report.

8. Special reporting requirements:

Nothing to report.

9. Appendix:

Revised SOW 08/15/18.

REVISED STATEMENT OF WORK – 08/15/2018
START DATE Sept 01, 2017

Site 1: Case Western Reserve University
11100 Euclid Ave
Cleveland, OH 44106
PI: Afshin Dowlati

Specific Aim 1: **To determine the expression and cellular localization of HEPACAM2 in SCLC:**	**Timeline** **(Months)**	
Major Task 1: To explore HEPACAM2 expression in SCLC and control cell lines (*ALL commercially available*: **SCLC** H1694, H211, H1092, H1048, SHP77, H2141, H209, H82, DMS114, SW1271, H841, H1184; **NSCLC** A549, SW900, H2009, H1299, H520; **NORMAL** NL20)		
Subtask 1: Perform Taqman qPCR on: 1a. Endogenous HEPACAM2 1b. siRNA-treated SCLC to transiently KD HEPACAM2 1c. FLAG-tagged cells over-expressing HEPACAM2 1d. Cells with CRISPR KO HEPACAM2	1-18	Karen McColl
Subtask 2: Perform western blots on protein lysates from: 2a. Endogenous HEPACAM2 2b. siRNA-treated SCLC to transiently KD HEPACAM2 2c. FLAG-tagged cells over-expressing HEPACAM2 2d. Cells with CRISPR KO HEPACAM2	3-18	Karen McColl
Subtask 3: Perform confocal microscopy.	18-24	Karen McColl, Microscopy core
Major Task 2: To analyze HEPACAM2 expression in SCLC tumors:		
Subtask 1: Optimize IHC detection of HEPACAM2 in SCLC tumors. (*performed on cell line pellets or slides, named above*)	18-20	Histology core
Subtask 2: IHC stain 2-3 SCLC TMAs for HEPACAM2. (*in-house made TMAs*)	20-22	Histology core
Subtask 3: Read & correlate IHC staining with clinical outcomes.	22-24	Dr. Yang, Dr. Fu, Dr. Dowlati
Milestone(s) Achieved: IRB approval for TMA study	*02/28/2018 (most recent, good for 1 yr)*	
Milestone Achieved: HRPO/ACURO Approval:	*09/15/17 (original) 06/07/18 (continuing review approval)*	

Specific Aim 2: **To determine if SCLC cells demonstrate increased sensitivity to TNKS inhibitors and the phenotype of HEPACAM2 OE/KD cells:**		
Major Task 1: Determine efficacy of TNKS inhibitors in SCLC and NSCLC cells as controls: (subset of cells named above)		
Subtask 1: Determine efficacy on cell growth, adhesion & migration using IncuCyte ZOOM.	6-24	Karen McColl
Subtask 2: Determine effects on subcellular localization.	6-24	Karen McColl, Microscopy core
Major Task 2: Determine effect of HEPACAM2 over-expression and knockdown: (subset of cells named above)		
Subtask 1: Determine effects on cell growth, adhesion & migration using IncuCyte ZOOM.	12-24	Karen McColl, Dr. Dowlati
Subtask 2: Determine effects on subcellular localization.	12-24	Karen McColl, Dr. Dowlati
Milestone(s) Achieved:		