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TITLE: RNA Degradation Enzymes: Novel Targets in Non-Small Cell Lung Cancer

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CONTRACTING ORGANIZATION: Yale University, New Haven, CT

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14. ABSTRACT

Lung cancer is the number one cause of cancer death in the United States, and more than 80 percent of lung cancers are non-small cell lung cancer (NSCLC). Prognosis of the majority of NSCLC patients remains poor. Recently, a new group of cancer drugs, that, instead targeting the tumor cells, target the immune cells in the tumor environment, have shown unprecedented success in the treatment of NSCLC patients. These findings suggest that targeting the tumor environment, rather than the tumor cells themselves, may be a successful strategy. Vascular endothelial cells in the tumor, or tumor endothelial cells (TECs), are important components of the tumor environment. TECs harbor a number of molecular and structural abnormalities and help the tumor grow and propagate in various ways. In this proposal we will study the utility of targeting a specific molecular abnormality in TECs. We have previously found that a small tumor suppressor gene, called microRNA-1 (miR-1), in TECs is important for tumor growth. In NSCLC patients the level of miR-1 is lower in TECs compared to the non-cancerous tissue, and patients with higher levels of miR-1 in their tumors survive longer compared to patients with lower levels. Also, raising miR-1 levels in animal models of NSCLC through artificial methods stops tumor growth.

15. SUBJECT TERMS
None listed.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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1. **INTRODUCTION:**

Tumor endothelial cells (TECs) are critical components of the tumor microenvironment and chronic simulation by angiogenic cytokines, such as vascular endothelial growth factor (VEGF) keeps them in a dysfunctional, chronically "activated" state. In this proposal we will study the clinical and pathophysiological significance of a molecular abnormality in TECs that is induced by VEGF and affects the levels of microRNA-1 (miR-1). We had previously shown that miR-1 plays a critical regulatory role in TECs. MiR-1 levels are lower in TECs, compared to the adjacent non-cancerous tissues; and VEGF decreases miR-1 levels in the primary endothelial cells. Overexpression of miR-1 in the tumor endothelium of murine NSCLC models decreased tumor burden and vascularity. In our preliminary studies we found that VEGF downregulates endogenous mature miR-1, without altering its biogenesis. In small RNA sequencing VEGF stimulation led to the emergence of unique 3prime-adenylated and trimmed miR-1 isoforms (isomiRs), suggesting that 3prime-adenylation triggers miR-1 degradation. Since it is recently discovered that polyA polymerase domain containing enzymes, (PAPDs) mediate the 3prime-adenylation of miRNAs, we have hypothesized that: **1-PAPDs regulate miRNA levels in the tumor endothelium by triggering their degradation.** And that **2-PAPDs control the growth and propagation of NSCLC tumors.** We will test the validity of our hypotheses in the following aims:
Aim1: We will characterize the role of PAPDs in miRNA adenylation and degradation in the tumor endothelium. We will use Time-lapse sequencing method, to compare the rates of miR-1 degradation in TECs vs controls and determine the effect of PAPD knockdown and overexpression on those rates.
Aim2: We will determine the role of PAPDs in NSCLC tumor progression in the KRAS/P53 model of NSCLC: We will test the effects of PAPD knockdown and overexpression on tumor growth, immunity and vascularity in KRAS mutant/P53 knockout mice.
Aim3: We will determine the predictive value of PAPDs for tumor progression in NSCLC patients: We will use tissue microarray (TMA) technology and Cox proportional hazard (CPH) regression to assess the value of PAPD enzymes for predicting the overall survival in our 300-patient NSCLC cohort.

2. **KEYWORDS:**

Non small cell lung cancer

Tumor endothelial cells (TECs)

MicroRNA (miRNA)

RNA 3' adenylation

RNA degradation

Tumor progression

3. **ACCOMPLISHMENTS:**

- **The major goals of the project:**

Specific Aim 1: To determine the role of PAPDs in miRNA adenylation and degradation in the tumor endothelium:
Subaim 1a: To determine the rate of mature miRNA adenylation and degradation in tumor endothelial cells.
Subaim 1b: To determine the role of PAPDs in miRNA adenylation and degradation.
Specific Aim 2: To determine the role of PAPDs in NSCLC tumor progression through the use of the lentiviral vectors described in aim1a to knockdown, or overexpress PAPD proteins in the KRAS/P53 model.
Specific Aim 3: To determine the predictive value of PAPDs for tumor progression in NSCLC patients by using tissue microarray (TMA) technology and Cox proportional hazard (CPH) regression to assess the clinical correlations of PAPD4 and 5 and their value as predictors of tumor progression

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

Aim1:
Major activities**: To determine the role of PAPDs in miRNA adenylation and degradation in the tumor endothelium**

Specific objective: To determine the rate of mature miRNA adenylation and degradation in tumor endothelial cells In this aim we had planned to determine the basic rate of adenylation and degradation in TECs. We had planned to start these experiments on TECs that were isolated from patient samples. These cells were stored in our freezers during the pandemic, and unfortunately, we have not been successful at propagating them in culture after returning to the lab. We think that this issue may be due to heat shock after a couple of episodes of power outage episodes. Nevertheless, following the same plan as described in the proposal we have performed three activities in this aim:

Key outcomes:

1- Human lung microvascular endothelial cells (HLMECs): Following the suggestion of our grant reviewers, we obtained and started working with) and found that these cells also respond to VEGF by downregulating miR-1. (data not shown)

2- Isolation and validation of TECs: We have obtained new lung tumor and adjacent tissues from patients going through surgical resection at Yale, and isolated endothelial cells from tumors and adjacent non-cancerous lung tissue, using anti-CD31-biotin and streptavidin-magnetic beads (Miltenyi Biotec), as described in the proposal and validated the identity and purity of these cells by staining for vascular-specific markers, CD31 (PECAM-1), and Von Willebrand factor (VWF). (Figure 1, panel a and b)

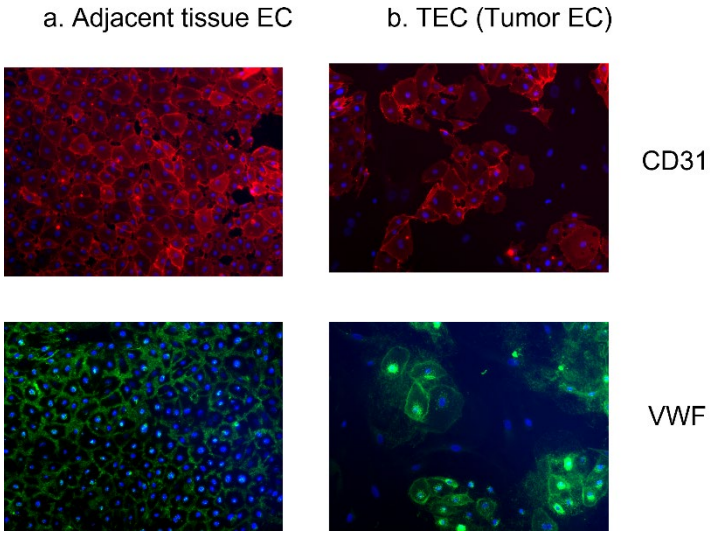

Figure 1. ant-CD31 and anti-VWF staining of newly isolated endothelial cells (ECs) from tumors and adjacent (non-cancerous) tissues.

3- Optimization of Time lapse sequencing for tumor endothelial cells: We used HUVEC-Bcl2 cells, a known model of tumor endothelial cells (Nör, Christensen et al. 2001) to set up the basic steps of VEGF stimulation and 4-thiouridine (s4U) incorporation for time-lapse sequencing. Using these cells, we showed that VEGF stimulation causes miR-1 levels to decrease by more than 80% and PAPD4 levels to increase more than twice its basal levels after 12 hours (Figure 2a and b respectively).

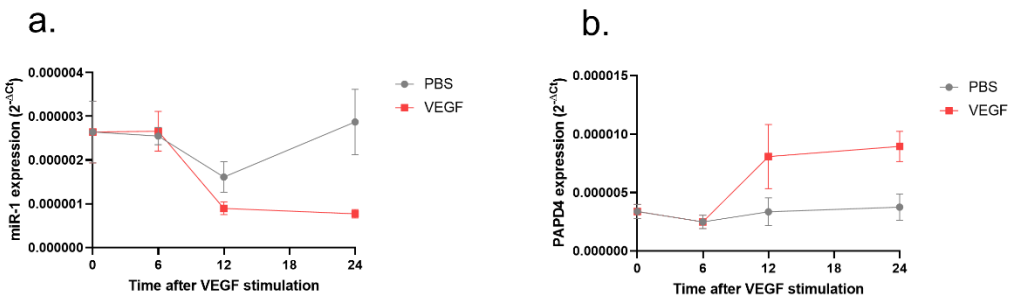

Figure 2: HUVEC-bcl2 cells were stimulated by adding VEGF (100 ng/ml) to media and cell aliquots were removed at 4, 12, and 24 hours after stimulation and cellular were isolated. The levels of mature miR-1(a) and PAPD4 mRNA (b) were measured by performing qRT-PCR and expressed as 2-Δct)

We next tested whether s4U would get incorporated into the endothelial cells after its addition to the culture media and whether adding VEGF would enhance this uptake. In time-lapse sequencing, newly produced RNA will be isolated by first incorporating s4U-botin into the RNA using methylthiosulfonate (MTS) chemistry and then isolating this biotinylated RNA and sequencing it. To mimic these experimental conditions, we used MTS-TAMRA to fluorescently label RNA and quantify s4U incorporation by dot-blotting, as described before.(Duffy, Rutenberg-Schoenberg et al. 2015) As shown in figure 3, s4U is incorporated into HUVEC-bcl2 at a very low level at baseline, but VEGF stimulation causes a 12-fold increase in the incorporation rate.

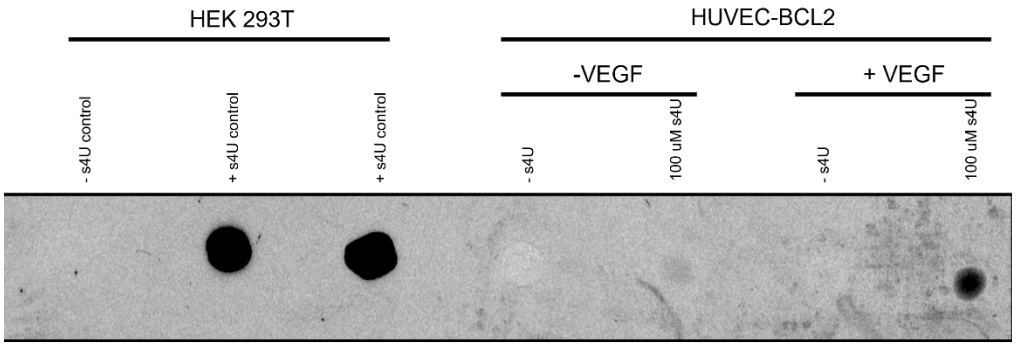

Figure 3: optimization of 4-thioU incorporation using MTS chemistry: HUVEC-bcl2 and HEK293T cells were cultured in the presence or absence of 100 uM s4U-TAMRA. VEGF at the final concentration of 100 ng/ml was added to the test group of HUVEC -bcl2 cells. Cells were harvested after 12 hours, RNA isolated and the incorporation of the fluorescent s4U quantified after dot-blotting. The top panel shows the dot blot with each condition. HEK 293T cells were used as controls and were blotted at two different concentrations. The table shows the concentration of the RNA used for blotting and the total fluorescence intensity in each sample.

Sample	Concentration (ng/uL)	TAMRA Fluorescence Intensity
293T, -s4U ctrl.	51.3	NA
293T, +s4U ctrl.	122.2	21,663.45
293T, +s4U ctrl.	66.1	14,603.50
HUVEC, -s4U	10.3	NA
HUVEC, 100 uM s4U	4.8	533.58
HUVEC, VEGF, -s4U	24.1	NA
HUVEC, VEGF, 100 uM s4U	32.8	6965.92

Aim2:

Major activities: **To determine the role of PAPDs in NSCLC tumor progression.**

Specific objective: To test the effects of PAPD knockdown and overexpression on tumor growth, immunity and vascularity in KRAS mutant/P53 knockout mice.

Significant outcome: PAPD4 and PAPD5 knockdown in KRAS mutant-P53 knockout (KP) mouse: this part of the grant is being performed at two sites: yale and MD Anderson.

-At Yale: We restarted the breeding of our KP colony, genotyped and selected the positive mice, and set up an experiment with the PAPD4 knockdown vector described in the proposal. In these mice we induced tumor formation by delivering the Cre vector and then inhibited PAPD4 expression by delivering the knockdown vector 4 months after Cre delivery. The first batch of the mice from this experiment were sacrificed recently and figure 4 shows representative images of their lungs and levels of miR-1 and PAPD4 measured in the whole lung lysate. The number of mice in this group is not adequate for statistical significance but the measurements show a trend toward lower levels of PAPD4 in the knockdown group and higher levels of miR-1 I this group, as expected.

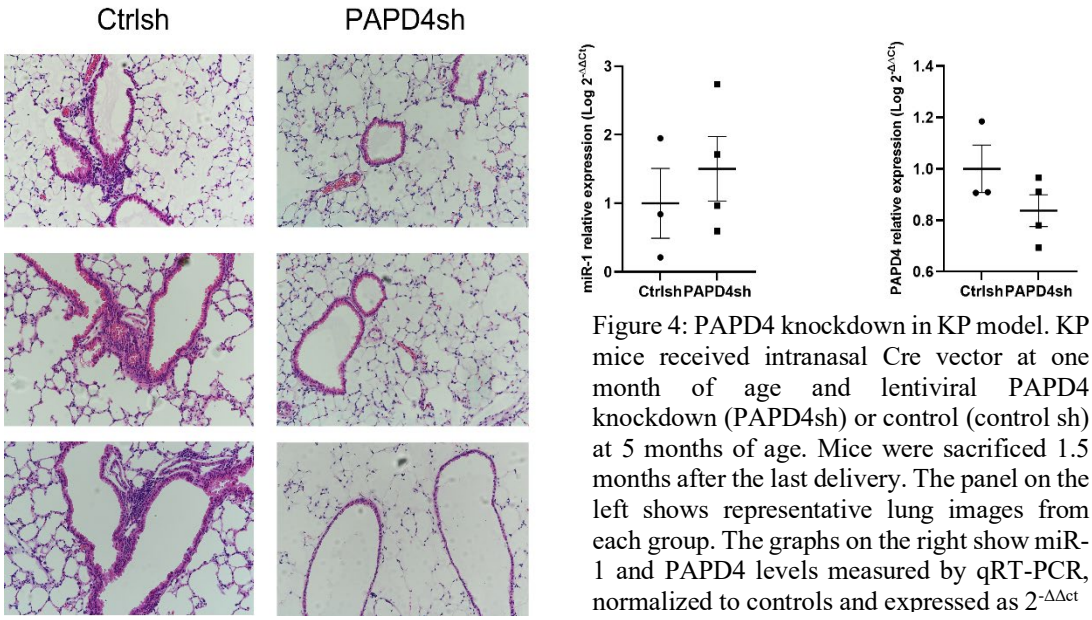

Figure 4: PAPD4 knockdown in KP model. KP mice received intranasal Cre vector at one month of age and lentiviral PAPD4 knockdown (PAPD4sh) or control (control sh) at 5 months of age. Mice were sacrificed 1.5 months after the last delivery. The panel on the left shows representative lung images from each group. The graphs on the right show miR-1 and PAPD4 levels measured by qRT-PCR, normalized to controls and expressed as $2^{-\Delta\Delta ct}$

-At MD Anderson: the experiment has been started with both PAPD4 and PAPD5 vectors that we delivered to their lab. In the first batch they have 3 groups (PAPD4 knockdown, PAPD5 knockdown, and control) and 3 mice per group. The Cre gene has been induced and mice have received the respective vectors and are going to be sacrificed in the week of November 29th.

Aim3:
Major activities: **To determine the predictive value of PAPDs for tumor progression in NSCLC patients**

Specific objective: To assess the clinical correlations of PAPD4 and 5 and their value as predictors of tumor progression using tissue microarray (TMA) technology and Cox proportional hazard (CPH) regression.

Significant outcomes: Preparation of tissue microarray from NSCLC patient samples and optimization of the antibody for detection of PAPD4 levels in tissue microarray: According to the plan, a tissue microarray was prepared from 200 NSCLC patients' tumor and adjacent tissue (stroma) samples and 20 normal (non-cancerous) lung samples by Yale Pathology Tissue Services (YPTS) / Tissue Microarray Facility. We optimized a commercial anti-PAPD4 antibody (Abcam cat# ab103884) for 3,3'-Diaminobenzidine (DAB)-based immunoperoxidase staining in histopathology samples and submitted the antibody and our protocol to YPTS for staining. The staining and analysis were done according to the method described in the proposal. As shown in figure 5, the preliminary analysis of PAPD4 expression showed that, as expected, PAPD4 levels were higher in the tumors, compared to the stroma, and in tumors compared to the normal (non-cancerous) lung samples.

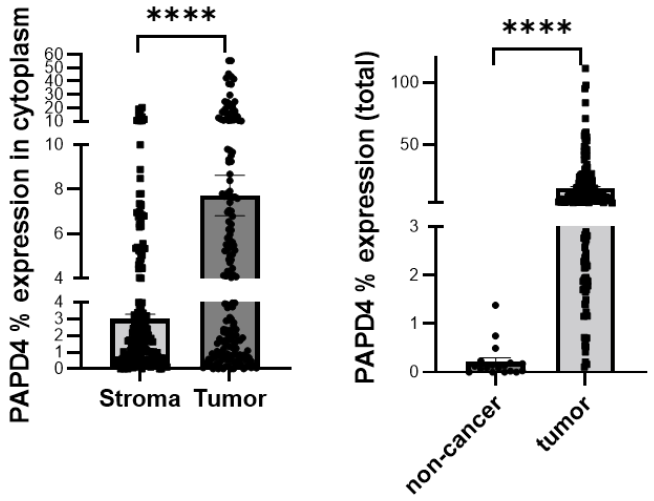

Figure 5: PAPD4 expression on NSCLC Tissue microarray: Tissue microarray slides with tumor samples and non-cancer lung samples were assessed for PAPD4 protein expression using chromogenic immunohistochemistry with anti-PAPD4 antibody (1:25dilution). AQUA (Automated Quantitative Analysis) was used as a primary method for quantifying PAPD4 expression in cells. To validate automated analysis, immunostained slides were evaluated by a pathologist at Yale University. (Tumor microarray, n=200 NSCLC patients, ****p<0.0001 and non-cancer tissue microarray, n=20 subjects, ****p<0.0001).

- **What opportunities for training and professional development has the project provided?** *"Nothing to Report."*
- **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?**

In the next phase of the study:

***In Aim1**, we will:*

1-Use HLMECs and TECs (isolated from NSCLC patients and validated, figure 1) in the S4U incorporation experiment and perform the timelapse sequencing to detect the levels of miR-1 and its isomiRs.

2-Treat these cells with PAPD enzyme knockdown and overexpression vectors and check the rate of miR-1 degradation by timelapse sequencing

***In Aim2**, we will:*

1-Finish the current KP mouse experiment with PAPD4 and PAPD5 knockdown lentiviral vectors (the latter is being performed in MD Anderson by our collaborator, Dr. Moghadam) and analyze the lungs for tumor burden, vascularity, and inflammatory markers, as described in the proposal.

2-We have constructed a vascular-specific PAPD4 lentiviral vector for this experiment. Following the recommendation of our reviewer, we will use this vector in a KP mouse experiment similar to our current study.

***In Aim3**, we will:*

1-Assess the correlation of PAPD4 levels with overall survival and other clinical characteristics of NSCLC patients in our cohort.

2-Use Cox proportional hazard modeling to assess the utility of PAPD4 and PAPD5 levels as predictors of tumor progression.

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*
    - **What was the impact on the development of the principal discipline(s) of the project?** *"Nothing to Report."*
    - **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?** *"Nothing to Report."*
5. **CHANGES/PROBLEMS:** *"Nothing to Report,"*
6. **PRODUCTS** *"Nothing to Report."*
7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**
    - **What individuals have worked on the project?**
        -

Name:	*Seyedtaghi (Shervin) Takyar*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.0
Contribution to Project:	As PI, Dr. Takyar oversees all aspects of these studies and ensures the overall success of this project, from coordinating analyses with the research team to ultimately publishing the results
Funding Support:	

Name:	*Matthew Simon*
Project Role:	*Co-Investigator*

Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.0
Contribution to Project:	Dr. Simon contributed to this project by designing and taking part in the performance of experiments for optimization of 4SU incorporation into primary endothelial cells.
Funding Support:	Matt Simon receives summer compensation during the summer months of June-August. Summer compensation is not his academic salary but it is in addition to his academic salary.

Name:	*Asawari Korde*
Project Role:	*Postdoctoral Associate*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12.0
Contribution to Project:	Dr. Korde performed the molecular biology experiments including cell culture, expression studies, library preps for RNA sequencing, RNA interference studies, DNA plasmid cloning, cytokine cell signaling, cell culture and confocal microscopy under the supervision of Dr. Takyar.
Funding Support:	

Name:	*Seyed Javad Moghaddam*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.0
Contribution to Project:	Dr. Moghaddam oversees all aspects of the proposed study that are performed at MD Anderson including assessment of lung tumor development, immune profiling, temporal and carcinogenesis experiments in mice, analysis of the interplay between tumor cells and pro-tumor inflammatory pathways due to the proposed intervention.
Funding Support:	

Name:	*Marco Ramos*
Project Role:	*Research Assistant II*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6.0

Contribution to Project:	Mr. Ramos is directly in charge of conducting experiments in Dr. Moghaddam's laboratory at MD Anderson. This includes animal maintenance, genotyping, intervention and treatment, dissection and tissue harvesting, lung tumor evaluation, cell and molecular analysis such as immunophenotyping of immune cells in bronchoalveolar lavage fluid (BALF), RNA and protein based analysis of BALF (supernatant, and cell pellet) and frozen lung tissue, as well as lung immunohistopathologic analysis. He also works with Dr. Moghaddam in data analysis, figure and graph preparation.
Funding Support:	

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

New Active Support

2 new grants began since the onset of this grant, one where Dr. Takyar is PI, another where Dr. Takyar is Co-I:

Supported Studies (Takyar) 07/01/20 – 06/30/22
GSK Pharmaceuticals
An endothelial microRNA mediates the IL-5 effect on airway eosinophilia
The goals of this project are to determine the effect of Mepulizumab on miR-1 and the importance if this pathway in eosinophil trafficking and to determine the utility of miR-1 as a predictor of the response to Mepolizumab. Role: PI

1 R01 HL153604-01 (Gomez) 07/01/20 – 06/30/25
NIH/NHLBI
Systems Biology of a MicroRNA Network in Neutrophilic Asthma
The goals of this study are to determine the longitudinal expression of the sputum miRNA network and its role in neutrophilic airway inflammation, define the network miRNAs released in extracellular vesicles and their association with neutrophilic airway inflammation, and determine the effect of the miRNA network in airway epithelial gene expression and its contribution to neutrophilic airway inflammation. Role: Co-I

- **What other organizations were involved as partners?**
  - **Organization Name:** MD Anderson Cancer Center at University of Texas
  - **Location of Organization:** Houston, TX
  - **Partner's contribution to the projec**t *(identify one or more)*
    - **Collaboration** *(e.g., partner's staff work with project staff on the project)*;

Dr. Seyed Moghaddam oversees all aspects of the proposed study that are performed at MD Anderson including assessment of lung tumor development, immune profiling, temporal and carcinogenesis experiments in mice, analysis of the interplay between tumor cells and pro-tumor inflammatory pathways due to the proposed intervention.

Duffy, E. E., et al. (2015). "Tracking Distinct RNA Populations Using Efficient and Reversible Covalent Chemistry." Mol Cell **59**(5): 858-866.

We describe a chemical method to label and purify 4-thiouridine (s(4)U)-containing RNA. We demonstrate that methanethiosulfonate (MTS) reagents form disulfide bonds with s(4)U more efficiently than the commonly used HPDP-biotin, leading to higher yields and less biased enrichment. This increase in efficiency allowed us to use s(4)U labeling to study global microRNA (miRNA) turnover in proliferating cultured human cells without perturbing global miRNA levels or the miRNA processing machinery. This improved chemistry will enhance methods that depend on tracking different populations of RNA, such as 4-thiouridine tagging to study tissue-specific transcription and dynamic transcriptome analysis (DTA) to study RNA turnover.

Nör, J. E., et al. (2001). "Up-Regulation of Bcl-2 in microvascular endothelial cells enhances intratumoral angiogenesis and accelerates tumor growth." Cancer research **61**(5): 2183-2188.

8. Special Reporting Requirements.

Nothing to Report

9. Appendices

Not applicable