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TITLE: Mechanisms of Metastasis Suppression and Translational Applications in Thyroid Cancer

PRINCIPAL INVESTIGATOR: Matthew D. Ringel, MD

CONTRACTING ORGANIZATION:
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14. ABSTRACT
Progressive metastases are responsible for most deaths from thyroid cancer. We have identified that loss of RCAN 1.4 is a key event in metastatic progression due to increased NFE2L3-mediated IL-8 expression and by inducing a tumor-promoting immune environment. The IL-8 system is not able to be studied in mouse models and the pace of translation to define regulatory mechanisms regulating metastatic progression in immune competent systems requires arduous mouse models that delay translational efficiency. To test the role of IL-8 and RCAN1.4 loss and to model the tumor: immune interface in a human cell based model with more rapid translational potential, we will develop a highly reproducible thyroid cancer metastasis on a chip platform that will enable mechanistic studies of the RCAN 1.4 metastasis suppression pathway, create a new platform to study mechanistic regulation of the tumor-immune interface, and allow for more rapid validation of biomarkers and therapeutic targets.

15. SUBJECT TERMS: Thyroid Cancer, Metastasis, Metastasis-on-a Chip, Tumor Immunology, Metastasis Suppression, Lung Metastasis

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Introduction:

Progressive metastases are responsible for most cancer deaths, including from thyroid cancer. In human thyroid cancers, and using cell lines in two dimensional (2D) tissues and in mice, we identified that loss of expression of the regulator of calcineurin 1.4 (RCAN 1.4) is a key event in metastatic progression. Mechanistic studies demonstrated that overexpression of the transcription factor NFE2L3 is responsible for the pro-metatastatic impact of RCAN 1.4 loss and that this occurs in part via enhanced IL-8 expression leading to a tumor-promoting immune environment. However, the IL-8 system is not able to be studied in mouse models as this protein is not expressed, and the mechanisms for the immune changes are not certain. Standard 2D *in vitro* or *ex vivo* systems to study cancer invasion or three dimensional (3D) growth do not recapitulate metastasis. To study the role of IL-8 and to model the tumor:immune interface in a human metastatic model with more rapid assays vs mouse models, our goal was to first develop a reproducible bioengineered metastasis-on-a-chip (MOC) platform for metastatic thyroid cancer using human cells to enable mechanistic studies of this pathway. Development and validation of this system also will create a novel and innovative system to not only study the tumor-immune interface, but also enable rapid validation of biomarkers and therapeutic strategies targeting metastatic progression.

Keywords

Thyroid Cancer, Metastasis, Metastasis-on-a-Chip, Organoid, Tumor Immunology, Cancer Progression, Metastasis Suppressor, RCAN1.4, NFE2L3, IL8 (CXCL8), Lung Metastasis

Accomplishments

Specific Aims:

- **What were the major goals of this project:**

<u>Major Task 1:</u> Pre-Research Start: Completed

<u>Specific Aim 1: Develop Metastasis-on-a-Chip for RCAN 1.4 regulated Thyroid Cancer</u>
<u>Major Task 2</u> – Biofabrication of a thyroid cancer MOC (tcMOC): 12 months planed completion: Completed 100% (6/30/2023) milestone of biofabricated the microfluidic device and integrating organoids and metastasis-on-a-chip models using thyroid cancer cells and lung metastasis model.

<u>Major Task 3</u> – Quantification of metastasis and invasion kinetics with and without RCAN1.4 knockdown: 18 months planned completion: 70% completed: Subtask 1 is completed, subtask 2 is ongoing.

<u>Specific Aim 2: Determine the roles of immune populations in RCAN 1.4 invasion and metastases</u>
<u>Major Task 1</u> – Integration of immune cell components to the tcMOC: 24 months planned completion: 10%; subtask 1 ongoing.

<u>Major Task 2</u> – Evaluate effects of IL-8 and key identified cytokines on MDSCs, lymphocytes, and neutrophils: 30 months planned completion: 0% completed.

<u>Major Task 3</u> – Evaluate the relationship between RCAN 1.4 and loss of naïve T cells/T cell exhaustion: 36 months planned completion: 0% completed.

<u>Specific Aim 3 RCAN 1.4 loss-associated clinical validation</u>
<u>Major Task 1</u> – Evaluation of RNA sequencing data in paired normal and metastatic tissues: 48 months for completion: 15% completed: Subtask 1 ongoing.

<u>Major Task 2</u> – Potential Biomarkers to predict metastatic progression: 48 months for complete: 15% completed: Ongoing work with both subtasks.

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

Specific Aim 1: Develop Metastasis-on-a-Chip for RCAN 1.4 regulated Thyroid Cancer

Major Task 2 – Biofabrication of a thyroid cancer MOC (tcMOC): 12 months planned completion: Over the course of the first year we developed a biofabrication process for the thyroid cancer metastasis-on-a-chip (tMOC) system. Initial studies led to the biofabrication of collagen I – hyaluronic acid hydrogels to create primary thyroid cancers in the tMOC primary tumor chamber. Hematoxylin Eosin (H and E) staining confirmed the presence and growth of thyroid cancer cells lines with RCAN 1.4 knock-down (KD) and scrambled shRNA controls using two cell lines (two clones with one of the cell lines), as primary organoids in the hydrogel (Figure 1). This models a primary cancer. Western blot confirmed overexpression of NFE2L3 in the KD cells (hTh74>>FTC326; Figure 2) similar to previously published data (1). LIVE/DEAD IF staining confirmed that nearly all of the cells were viable (green vs red), and that the RCAN 1.4 KD cells tended to develop an elongated mesenchymal phenotype. In addition, we demonstrated the cells were proliferating using an ATP production assay, and that the proliferation was enhanced in the RCAN1.4 KD cells (Figure 2). Thus, we demonstrated that the primary organoids have very similar characteristics to the primary mouse xenografts from these cell lines that we previously published (1). We further confirmed biological similarity with the mouse xenograft model and human tumors by between the two systems by performing immunohistochemical (IHC) staining confirming that the hTh74 RCAN 1.4 KD thyroid cancer cells in the primary tumor model maintain overexpression of NFEL2.3 (Figure 3). Thus, we demonstrated that the tMOC primary tumor model maintained similar expression profiles to the mouse xenograft and *in vitro* studies using these same cells (1).

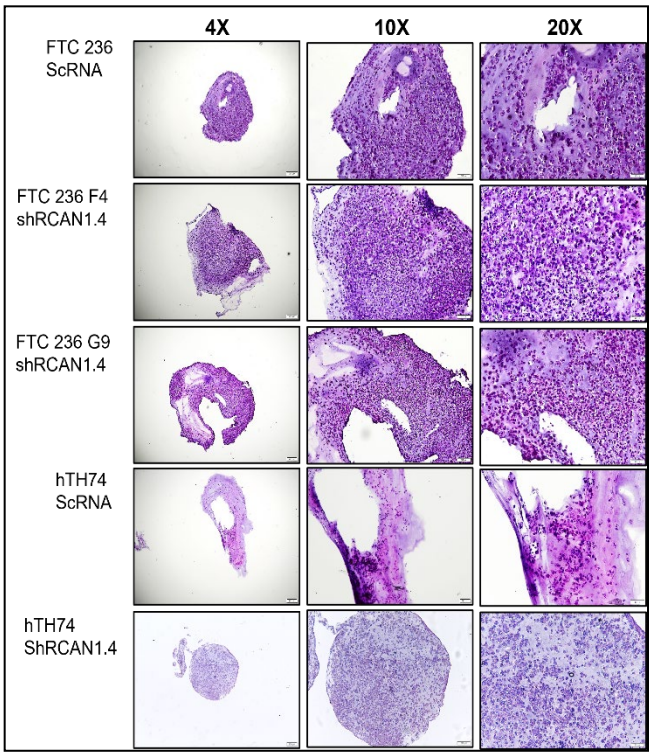

Figure 1. Thyroid cancer hydrogel (primary tumor model). H and E staining of the primary thyroid cancer cell in collagen I – hyaluronic acid hydrogels in the tissue scaffold demonstrating thyroid cancer cells (FTC 236 and hTh74) for cells expressing scrambled control (sc) shRNA and clones of FTC (F4 and G9) and hTh74 expressing shRNA for RCAN 1.4. Tumor cells are identified for all cell lines.

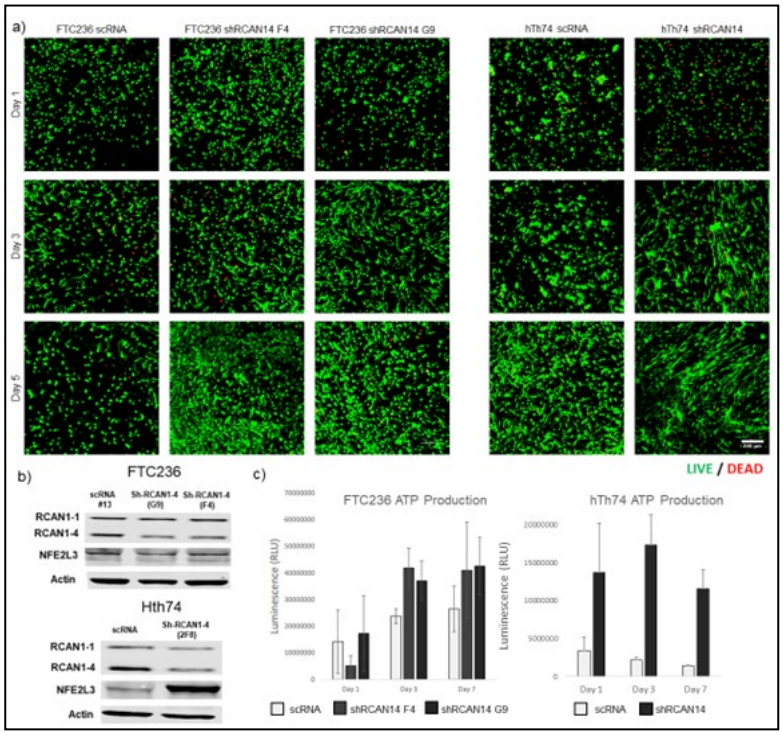

Figure 2. RCAN1-4 KD increases proliferation
a) Scrambled control (sc) and RCAN 1.4 KD clones of FTC236 and hTh74 cells grown in collagen I – hyaluronic acid hydrogels over five days show increasing cell density and little cell death. b) Western blot confirms RCAN1-4 knockdown independent of RCAN1-1 expression with a greater increase of NFE2L3 in hTH74 cells as previously published with these cells (1). c) RCAN1-4 KD induces higher quantitative proliferation by ATP measurement.

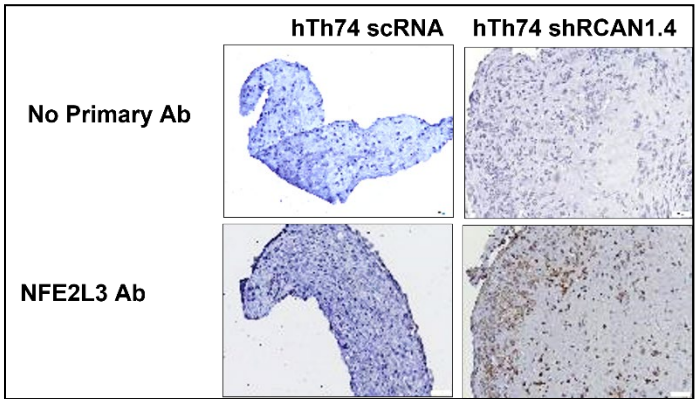

Figure 3: NFE2L3 expression is maintained in the primary thyroid cancer cell hydrogel. NFE2L3 IHC results confirm NFE2L3 expression (brown staining) which is most increased in hTh74 RCAN 1.4 KD cells.

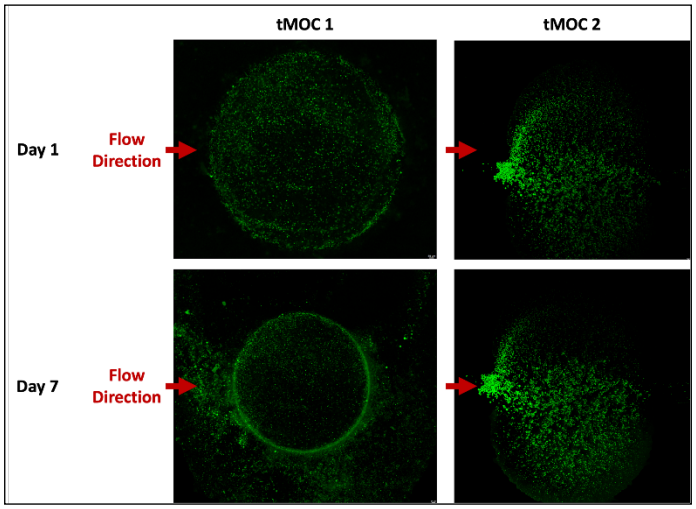

Figure 4: Optimization of the primary hydrogel tMOC. Two different tMOC conditions demonstrated local migration against interstitial flow prior to entering circulation using GFP-labeled FTC236 scRNA control cells.

The KD and control cell lines also stably express green fluorescent protein, which enables identification and monitoring of the cells in the tMOC system. Cells also can be detected using Mem-Glo label which contains a RFP as an alternative approach. Using the GFP expression, we assessed cell growth and invasion over time using different flow rates and orientation and optimized conditions for proliferation and invasion (Fig. 4, tMOC2). Notably, thyroid cancer cells in the primary organoid migrate against interstitial flow to exit the tumor organoid and enter circulation, after which they are able travel with the direction of circulation through tMOC device. This observation is important, as in cancer metastasis in human patients, the tumor cells typically also migrate against interstitial flow towards the blood vessels where that interstitial flow originates. We next developed prototypes for the tMOC model in which primary tumor cells are placed as a primary cancer organoid in one chamber, and a lung organoid using normal human lung bronchial epithelial cells and lung fibroblasts (Lonza) is in a second chamber connected by microfluidic channels. Proof-of-principle experiments using our initial configuration were performed first with the FTC236 control scrambled RNA and RCAN 1.4 KD cells (Figure 5a). As shown, after 14 days the control FTC236 cells establish the primary organoid and demonstrate the ability to enter the engineered vessels but do not seed or invade into a lung organoid. In contrast, the FTC236 ShRNA KD cells rapidly invade, enter the tMOC channels, seed, and invade into the lung organoids (5b). These experiments are currently being replicated using the modified structure in Figure 5b for all cell lines that was designed to enable increased throughput experiments. Thus, over the first 12 months of the award we have developed the full tMOC system for subsequent studies, confirmed

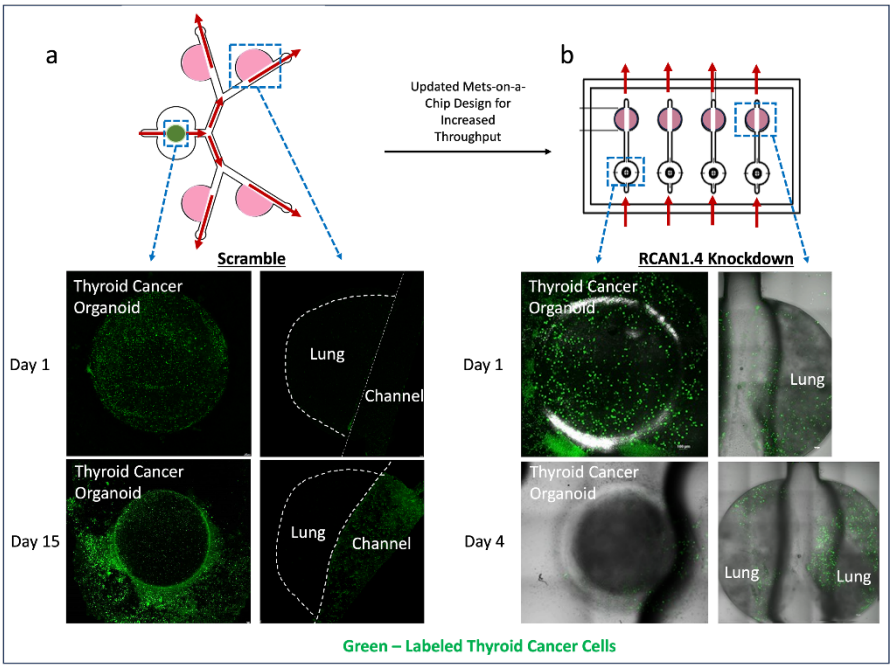

Figure 5: Schematics and proof-of-principle data from the tMOC. a) initial tMOC design and the application of FTC236 ScRNA control cells. These demonstrate primary tumor with invasion at days 1 and 15 with evidence of cells in the channel, but not in lung organoid. b) depicts data using the new increased throughput tMOC design using FTC236 RCAN1.4 KD cells with primary tumor invasion at day 1 and evidence of invasion into lung organoids as early as day 1 and day 4. Green stain – GFP-expressing thyroid cancer cells. Other cells in the lung organoids (lung fibroblasts and bronchial epithelial cells) are present, but not fluorescently labeled.

overexpression of NFE2L3 in the primary tumor model, and demonstrated that RCAN 1.4 KD results in increased proliferation and invasion with preliminary studies suggesting enhanced metastasis.

<u>Major Task 3</u> – Quantification of metastasis and invasion kinetics with and without RCAN1.4 knockdown: 18 months planned completion: Subtask 1 is completed, subtask 2 is ongoing. The cell lines we created are labeled with GFP for quantitation (Figures 4 and 5) and also can be detected and quantified using the Mem-Glo-RFP immunofluorescent dye. To confirm the ability to quantify metastasis and determine if this second assay system was a suitable alternative, the RCAN 1.4 KD cells and controls were injected into the tMOC channels and subsequent engraftment and invasion into the lung organoids were assessed 7 days after injection. This timing was determined based on initial time-course studies. Figure 6 demonstrates the quantitation of the distance of the leading edge movement of the invasive cells into the lung organoid. All of the RCAN1.4 KD clones invaded longer distances from the lung organoid edge versus control. Bulk invasion tended to be higher as well, but this is a measure both of cell adhesion and invasion. The distribution of cells moving longer distances was increased only for the hTh74 RCAN 1.4 KD cell line. The goal of this aim was to develop a system to both quantify and measure invasion over time, which we demonstrate and can utilize for subsequent experiments.

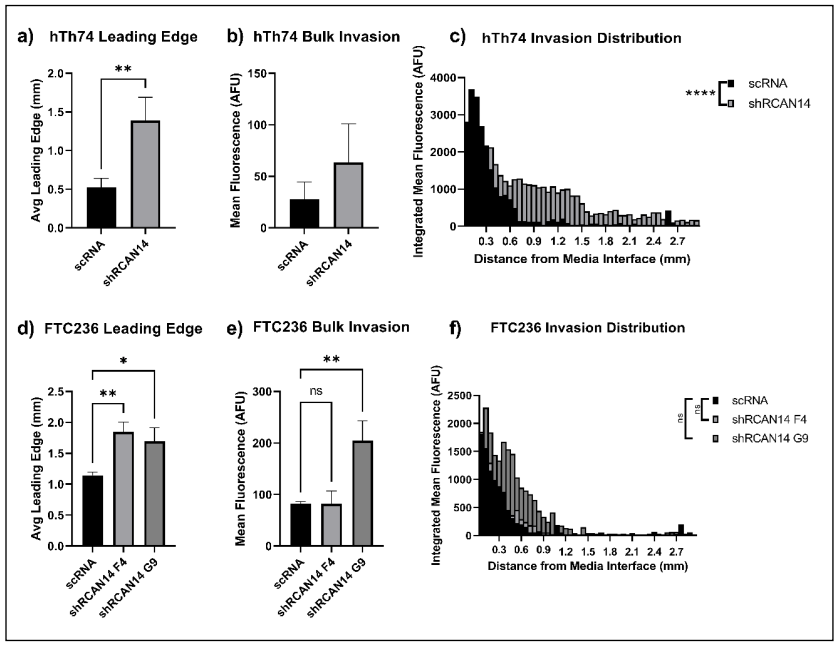

Figure 6. RCAN1-4 KD increases DTC attachment and invasion into lung hydrogel sites. RCAN1-4 KD (hTh74 and FTC236) clones and sc RNA control cells for each cell line were injected into engineered blood vessels and invasion into lung organoids was measured. RCAN1-4 KD hTh74 cells invaded into lung hydrogels significantly farther than scRNA control cells, as measured both by the distance of the average leading edge from the gel-media interface (a), and invasion distribution (c). b) RCAN1-4 KD did not significantly increase the ability of cells to adhere to and embed (bulk invasion) in lung hydrogels. d) In FTC236 cells, both RCAN1-4 KD clones invaded farther into lung sites vs. control cells. e) G9 RCAN1-4 KD had increased bulk invasion vs control while the F4 KD clone was not changed. f) Invasion distributions did not increase in either FTC236 KD clones. *p<0.05; **p<0.01; ****p<0.0001.

<u>Specific Aim 2 : Determine the roles of immune populations in RCAN 1.4 invasion and metastases</u>

<u>Major Task 1</u> – Integration of immune cell components to the tcMOC: 24 months planned completion: 10%; subtask 1 ongoing. We have identified sources of the white blood cells for experiments. While we have identified loss of naïve T cells, we have additional data in mouse models from studies performed separately from this award supporting an increase in M2 tumor macrophages in addition to the T cell changes. Thus, in addition to T cells, we will emphasize work on this population.

<u>Major Task 2</u> – Evaluate effects of IL-8 and key identified cytokines on MDSCs, lymphocytes, and neutrophils: 30 months planned completion: Planned to start once major task 1 is completed.

<u>Major Task 3</u> – Evaluate the relationship between RCAN 1.4 and loss of naïve T cells/T cell exhaustion: 36 months planned completion: These studies are planned for later in the grant cycle. Notably, we have established identification of a number of immune cells using mass cytometry (CyTOF) in mouse thyroid glands outside of this award and are optimizing a multiple antibody IF platform, the latter using antibodies that will be appropriate for human tissues. Thus, this work will facilitate the proposed work in this award.

<u>Specific Aim 3 RCAN 1.4 loss-associated clinical validation</u>
<u>Major Task 1</u> – Evaluation of RNA sequencing data in paired normal and metastatic tissues: 48 months for completion: We have evaluated and published that RCAN 1.4 is hypermethylated in intron 1 in human

thyroid cancer primary tumors vs normal tissue in associated with reduced RCAN 1.4 expression with an increase in NFE2L3 expression (2). These data are not funded by this award and they reflect the primary cancer only vs normal tissue. We have started RNA isolation from pairs of tumor and metastatic lesions for analysis as per the proposal.

Major Task 2 – Potential Biomarkers to predict metastatic progression: 48 months for complete:
15% completed: Ongoing work with both subtasks. We have expanded our number of tumors through the ORIEN registry to 885 individuals from which a subset have RNA sequencing and robust clinical data with tumor and normal tissue pairs linked to staging and follow up. This will enhance our ability to focus on NFE2L3 overexpression and relationships with immune infiltrates and clinical progression/outcomes as outlined in the proposal.

References:

1. Wang C, Saji M, Justiniano SE, Yusof AM, Zhang X, Yu L, Fernández S, Wakely P Jr, La Perle K, Nakanishi H, Pohlman N, Ringel MD. RCAN1-4 is a thyroid cancer growth and metastasis suppressor. JCI Insight. 2017 Mar 9;2(5):e90651
2. Khanal T, Rajan N, Li W, Liyanarachchi S, Ringel MD The RCAN1.4 Metastasis Suppressor Is Hypermethylated at Intron 1 in Thyroid Cancer. Thyroid. 2023 May 15. PMID: 37051697.

1. **Impact:** We have developed the proposed novel system of thyroid cancer metastasis to lung tissue that is comprised of normal lung alveolar and lung fibroblasts. This model will be used for subsequent cancer biology studies related to the immune microenvironment that have been proposed. Moreover, the system has been modified to enable higher throughput as noted in Figure 5, and once confirmed, can be potential used in primary human thyroid cancer samples.

2. **Changes/Problems:** The primary changes related to optimizing the model for higher throughput capacity. We modified slightly the design and demonstrate that the thyroid cancers metastasize to lung using this system (Figure 5). Our plan was to utilize bioprinters for the tMOC production. However, this design is more amenable to soft lithography molding using 3D printed device molds. This method was used to create identical and easily created tMOC scaffolds.

3. **Products:**
   - While not completed, we have begun work on further increasing the throughput of the MOC platform, while making it compatible with high throughput and high content drug screening. If successful, we expect to be able file a provisional patent based on this modified MOC design.
   - Our first manuscript based on the tMOC has been written and is in editing stages by the investigators. We expect to submit it for publication in August 2023.

4. **Participants & Other Collaborating Organizations:**

5. **What individuals have worked on the project?**

   a. Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Name:	Matthew D. Ringel
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0001-8672-3266 (ORCID)
Nearest person month worked:	1.5 CM
Contribution to Project:	Dr. Ringel is the overall PI for this award. He has led and supervised the work on the thyroid cancer cell biology, maintenance and growth of the thyroid cells, selection of the cells, analysis of IHC and H and E staining. He and Dr. Skardal have overseen the first manuscript and subsequent studies and his group has been leading the analysis of human tumor data for clinical correlations. He leads meetings every two weeks between the two groups and monitors progress along with making recommendations for experimental modifications.
Funding Support:	

Name:	Xiaoli Zhang
Project Role:	Co-I
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.35 CM
Contribution to Project:	Dr. Zhang serves the role as a supervisory faculty member focused on bioinformatics
Funding Support:	

Name:	Christopher Merkel
Project Role:	Research Technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	5.32 CM
Contribution to Project:	Mr Merkel performed cell biology experiments confirming RCAN 1.4 loss in the selected cell lines with shRNA versus controls and performed H and E and IHC staining. He also maintained the cell lines.
Funding Support:	

Name:	Sandya Liyanarachchi
Project Role:	Research Scientist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	5.69 CM
Contribution to Project:	Ms, Liyanarachchi served as the primary biostatistician for laboratory experiments, and analyzed whole exome and RNA sequencing data from clinical sources with Dr. Zhang for RCAN 1.4 and NFE2L3.
Funding Support:	

Name:	Aleksander Skardal
Project Role:	Co-PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-2138-2453 (ORCID)
Nearest person month worked:	1.5 CM
Contribution to Project:	Dr. Skardal has co-led the project with Dr. Ringel. Dr. Skardal's lab fabricates and operates the tMOC platforms. Dr. Skardal has also overseen the generation of the team's first manuscript that will be ready for submission for publication in August 2023. Dr. Skardal supervises the microfabrication work and the operation of the tMOC.
Funding Support:	

Name:	Kylie Nairon
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	4.00 CM

Contribution to Project:	Ms. Nairon has performed work focused on generating the tMOC platform and performing metastasis experiments, including tumor cell tracking and metastasis kinetics quantification.
Funding Support:	

Name:	Sydney Anderson
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2.88 CM
Contribution to Project:	Ms. Anderson has performed work focused on generating and characterizing thyroid cancer organoids and tMOC platforms.
Funding Support:	

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

a. Nothing to report

b. Matthew Ringel, MD:

i. R01 (p21 activated kinases in thyroid cancer) has entered no cost extension pending competing renewal proposal to NIH

ii. Over the past year, Dr. Ringel have been the PI of the Ohio State University Center for Clinical and Translational Sciences (NIH funded CTSA). I had previously been deputy director. My effort increased from 15% to 30% which also accounts for the reduction in support related to the prior award ending.

7. What other organizations were involved as partners?

a. Nothing to Report

b. Describe partner organizations - academic institutions, other nonprofits, industrial or commercial

8. Special Reporting Requirements: None

9. Appendices: N/A