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TITLE: Understanding and Targeting Breast Cancer Metastasis-Initiating Circulating Tumor Cells and Niches

PRINCIPAL INVESTIGATOR: Min Yu

CONTRACTING ORGANIZATION: University of Southern California, Los Angeles, CA

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Dr. Min Yu

E-Mail: minyu@med.usc.edu

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

UNIVERSITY OF SOUTHERN CALIFORNIA
U S C
3720 S FLOWER ST FL 3
LOS ANGELES CA 90007-4318

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

One of the biggest challenges in breast cancer is how to prevent and treat metastasis. Our main goal is to identify cell intrinsic and extrinsic metastasis-promoting mechanisms and develop novel therapeutics for targeting metastasis. We aims to investigate the molecular and physical properties of metastasis-initiating circulating tumor cells (CTCs), the unique properties of metastasis-supporting niches, and the mechanism of immune evasion in CTCs. This past year is the first year of this funding award and the COVID-19 pandemic significantly impacted our research activities in a negative way. Despite the challenges and unusual circumstances, we still made progress in analyzing CTC heterogeneity in morphology and transcriptomes at the single cell level in our existing patient-derived CTC lines, optimization of the spatial transcriptomic profiling protocols for PDX tissues, as well as investigation of the mechanisms of hypoxia-mediated inhibition of CTC intrinsic interferon and antigen presentation signals.

15. SUBJECT TERMS
None listed.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
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1. INTRODUCTION:

One of the biggest challenges in breast cancer is how to prevent and treat metastasis. Our main goal is to identify cell intrinsic and extrinsic metastasis-promoting mechanisms and develop novel therapeutics for targeting metastasis. In Aim 1, we will investigate the molecular and physical properties of metastasis-initiating circulating tumor cells (CTCs). In Aim 2, we will investigate the unique properties of metastasis-supporting niches. In Aim 3, we will investigate the mechanism of immune evasion in CTCs.

2. KEYWORDS:

Metastasis, Circulating tumor cells, tumor microenvironment, liquid biopsy, metastatic niche, hypoxia, immune evasion

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The major goals of the projects are:

1. To understand the molecular and physical properties of metastasis-initiating CTCs.
2. To Understand the properties of metastasis-supporting niches.
3. To target TME-mediated epigenetic memory in CTCs.

What was accomplished under these goals?

1) Major activities

This past year is the first year of this funding award and the COVID-19 pandemic significantly impacted our research activities in a negative way. Since middle of March in 2020, one month after starting our award, we have to stop our research activities due to university-wide and state-wide shelter-in-place order to reduce the COVID-19 pandemic rate. We have to stop the patient sample recruitment, sacrifice our animal models needed for the research, halt all ongoing research, freeze down cell lines, and work remotely for 3 months. In late June, the research activities on campus resumed at 30% capacity till late fall when it was raised to 50% capacity till now.

Despite the challenges and unusual circumstances, we focused our major activities in the following aspects:

1. Due to the fact that we couldn't recruit new patients because of the COVID-19 pandemic, we focused on analyzing our existing patient-derived CTC lines to investigate the heterogeneity in morphology and transcriptomes at the single cell level.
2. We used our collected PDX models and metastatic organs to identify dormant and overt metastasis, which will be picked and subjected for further RNA-sequencing analysis.
3. We performed single cell RNA-seq analysis of 2 brain metastasis samples and are in the process of analyzing the result.
4. We investigated the potential mechanisms of hypoxia in the TME for suppression of type I interferon (IFN) and antigen presentation (AP) pathways.

2) Specific objectives
   1. To understand the heterogeneity of CTCs in order to identify the metastasis-associated molecular, morphological and physical characteristics in CTCs.
   2. Identify the metastasis-promoting niches.
   3. Evaluate the mechanisms of inhibition of CTC intrinsic interferon and antigen presentation signals, which may provide novel understanding of immune evasion and ways to overcome it.

3) Significant results or key outcomes
   a. We have developed several live imaging analysis of single CTCs to evaluate the morphological features. We optimized mitochondria, cytoplasm, and nucleus staining in live single cells in order to obtain 3D measurement of those features in individual CTCs (**Figure 1**). The same cells are also saved for future single cell RNA-seq analysis.

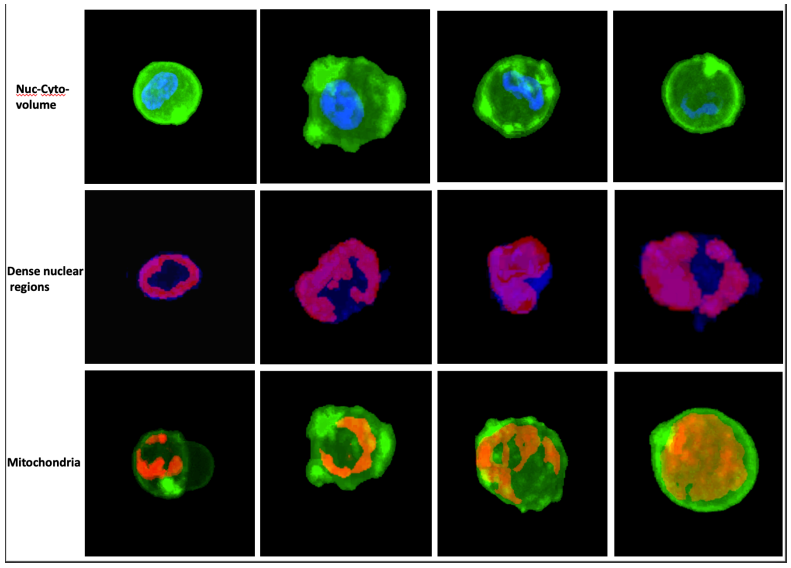
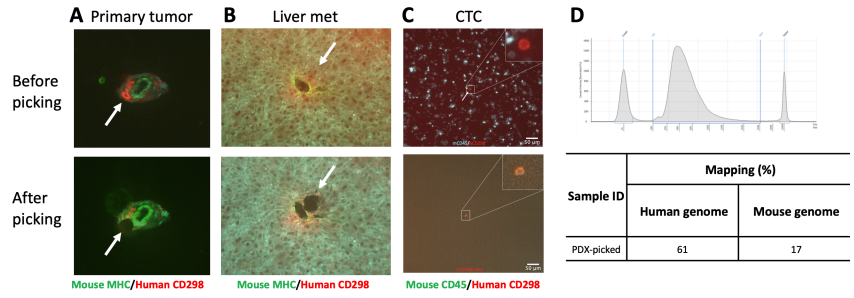


Figure 1. Representative images of single CTC staining to quantify nucleus-cytoplasm volume, dense nuclear regions, and mitochondria.

   b. We have optimized the fixation and reverse-crosslinking protocol for tissues that allow us to transcriptionally profile small regions of tissue. We have optimized a commercial CTC isolation system from RareCyte



Sample ID	Mapping (%)	
	Human genome	Mouse genome
PDX-picked	61	17

Figure 2. Spatial transcriptomic profiling. Representative image of before and after picking of single CTCs or small numbers of tumor cells from the primary tumor **(A)** liver metastasis **(B)** and blood circulation **(C).** The samples are stained with antibodies against human CD298 (red) and mouse MHC I (green) **(A-B)** or mouse CD45 (green) **(C)**. **D)** Representative Tapstation measure of cDNA library (top) and the mapping rate of the RNA-seq data (bottom) from the PDX picked region.

company to pick single CTCs, or small colonies (1-3 cells) of cells from fixed and immunostained tissues, and to obtain RNA to generate libraries for RNA-seq. Using breast cancer patient-derived xenograft (PDX) model, single cells or a small number of tumor cells adjacent to mouse blood vessels can be identified with a human-specific marker in primary tumor tissue, liver metastasis, and CTCs from the same mouse (**Figure 2A-C**). We have also modified fixing and staining protocols with reverse crosslinking to generate excellent cDNA libraries for high-quality RNA-seq analysis (**Figure 2D**). Via the RareCyte picking platform, approximately 1-3 tumor cells or stromal cells can be accurately picked for RNA-seq analysis.

c. Our recent work in CTCs and other tumor cells has revealed that hypoxia downregulates interferon (IFN) and antigen presentation (AP) pathways, and cells with downregulated IFN/AP pathways show enhanced tumor growth and metastasis (**Figure 3A-B**). We have identified an HIF-independent but PHD-dependent mechanism for hypoxia-mediated suppression of IFN/AP signal (**Figure 3C-D**). We used CRISPR/Cas9 technology to delete the obligated HIFα-cofactor, HIF1β (ARNT), and found that despite the loss of HIF activity in hypoxia, IFN/AP signals are still suppressed, suggesting an HIF-independent mechanism. A PHD inhibitor, FG4952, was able to mimic the hypoxic effect by reducing IFN target gene expression. To explore the existence of a "hypoxic memory" that confers advantageous characteristics to CTCs long after exposure to the hypoxic TME, we have generated a hypoxia inducible CRE reporter system. This reporter system converts RFP to GFP

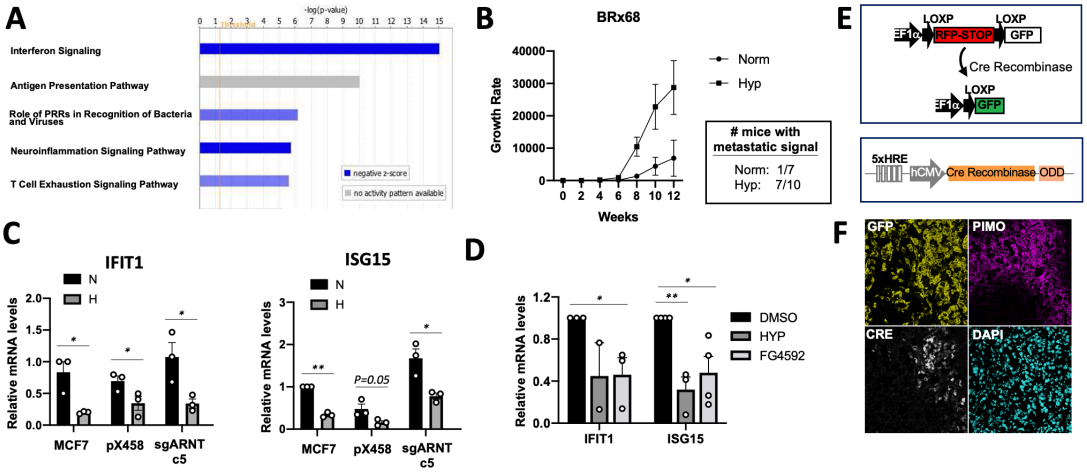


Figure 3. Hypoxia downregulates IFN/AP signaling and mediates long-term effects. A) Top canonical pathways enriched in genes differentially expressed in the BRx68 CTC line, cultured in normoxia or hypoxia (4% O_2). Graph shows -log(p-value). **B)** Hypoxic culture can promote primary tumor growth and metastasis formation. BRx68-GFP/luc cells cultured in normoxia or hypoxia for 8 weeks before injection into mammary fat pads for primary tumor growth or intravenously for metastasis. **C)** Relative mRNA expression levels of IFIT1 (top) and ISG15 (bottom) in bulk MCF7 cells, a control MCF7 clone and ARNT-KO MCF7 clone, grown in normoxia or hypoxia for 3 days. **D)** IFN targets IFIT1 and ISG15 expression levels in MCF7 cells treated with the PHD inhibitor FG4592 (100 mM) or hypoxia for 3 days. *P<0.05. **E)** The 2-plasmid design of the color-switching reporter system. **F)** Representative fluorescent images of a tumor section formed by MCF7 cells with the hypoxia tracing system stained with antibodies against GFP (green), pimonidazole (white) and Cre (red).

expression to trace cancer cells that have been exposed to hypoxic areas of the primary tumor (**Figure 3E-F**).

4) Other achievements

Nothing to report

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

The PI, Min Yu, has attended virtual meeting of AACR special conference on Cancer metabolism and epigenetics. This conference broadened her knowledge in cancer metabolism and its link with epigenetics.

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?

We plan to work with clinicians to recruit new blood samples from metastatic breast cancer patients to isolate, analyze CTCs and aim to establish more CTC lines. The paring of morphological and transcriptomic analysis at the single CTC level will help determine whether there are certain signaling pathways being reflected as unique morphological features. We also plan to continue the spatial transcriptomic analysis of the PDX models to identify transcriptional changes associated with various steps of metastatic cascade. For the evaluation of metastatic tumor and stromal cell interaction, we plan to continue the analysis of single cell RNA-seq for the brain metastasis samples, focusing on the ligand-receptor pairs between tumor and stromal cells. Moreover, we continue to investigate the mechanism and impact of hypoxia-mediated suppression of interferon and antigen presentation pathways in breast cancer cells.

4. IMPACT:

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:

Changes in approach and reasons for change

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

This past year is the first year of this funding award and the COVID-19 pandemic significantly impacted our research activities in a negative way. Since middle of March in 2020, one month after starting our award, we have to stop our research activities due to university-wide and state-wide shelter-in-place order to reduce the COVID-19 pandemic rate. We have to stop the patient sample recruitment, sacrifice our animal models needed for the research, halt all ongoing research, freeze down cell lines, and work remotely for 3 months. In late June, the research activities on campus resumed at 30% capacity till late fall when it was raised to 50% capacity till now. This year has significantly delayed our planned research progress, therefore we respectfully request to extend one additional year for completing our award. During the extension year, we hope to make up the lost time and effort toward completion of the proposed research.

Changes that had a significant impact on expenditures

Due to the impact of the COVID-19 pandemic as described above, our research activities are delayed, which lead to less cost than anticipated for this research year.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Nothing to report

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers and presentations.

Nothing to report

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

Nothing to report

- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Min Yu, PhD
Project Role:	PI
Researcher Identifier:	ORCID ID: 0000-0002-3969-8720
Nearest person month worked:	5.5
Contribution to Project:	Dr. Yu oversaw and directed all aspects of the project and supervised personnel.

Name:	Andrew Smith
Project Role:	Co-I
Researcher Identifier:	N/A
Nearest person month worked:	1.16 summer month

Contribution to Project:	Dr. Smith is an expert in applying computational methods to analyze large-scale genomic data sets. His computational biology research group designs the analytic technology required to leveraging massive and complex data sets that include various RNA-seq and ChIP-seq datasets. He has supervised data analysis and interpretation of results.
Name:	Keyue Shen
Project Role:	Co-I
Researcher Identifier:	N/A
Nearest person month worked:	1 summer month
Contribution to Project:	Dr. Shen has a strong background in researching the impact of stromal microenvironment at subcellular and cellular levels. His training background spans across multiple areas from mechanical engineering, micro-/nano fabrication, biomaterials, mathematic modeling, to cell biophysics, immunology, cancer bioengineering, and in vivo models. Particularly, his lab has generated devices to analyze tumor-stromal interactions and hypoxic devices for analyzing tumor microenvironments. He has assisted on analysis of the tumor-stromal interaction and interpretation of results.
Name:	Mohamed Saleh, PhD
Project Role:	Research Associate
Researcher Identifier:	N/A
Nearest person month worked:	10
Contribution to Project:	Dr. Saleh is the main person who developed the protocol for staining, scanning and picking live CTCs on RareCyte. He has been the core user for the RareCyte system. In this proposal, he has carried out the experiment to analyze patient CTCs using RareCyte as proposed in Goal 1. In addition, he performed the experiments proposed in Goal 1C on micrometastasis and dormant DTC analysis
Name:	Oihana Iriondo, PhD
Project Role:	Research Associate
Researcher Identifier:	N/A
Nearest person month worked:	3.5
Contribution to Project:	Dr. Iriondo generated the preliminary results for the hypoxia part of project in Goal 3. She has been the main person, working together with other collaborators, to perform animal, molecular, and sequencing experiments in the research goal 3.
Name:	Remi Klotz, PhD
Project Role:	Research Associate

Researcher Identifier:	N/A
Nearest person month worked:	1.7
Contribution to Project:	Dr. Klotz is the main person who generated the preliminary results used for tissue tropism part of the project in research Goal 1. He has carried out experiments in the Goal 1 (1A) and Goal 2 (2B) of this proposal, including cell culture, molecular biology analyses, and animal experiments. He also helped supervise graduate students and undergraduate volunteers.

Name:	Amber Dickerson
Project Role:	Research Lab Technician
Researcher Identifier:	N/A
Nearest person month worked:	3
Contribution to Project:	Ms. Dickerson has assisted the research teams in the animal experiments and other in vitro experiments

Name:	Diane Kang
Project Role:	Graduate Student
Researcher Identifier:	N/A
Nearest person month worked:	1.6
Contribution to Project:	Ms. Kang participated in analyzing the physical properties of the metastatic CTCs (Goal 1B) and tumor-stromal interaction for the lung metastasis (Goal 2B).

Name:	Veronica Ortiz
Project Role:	Graduate Student
Researcher Identifier:	N/A
Nearest person month worked:	1.3
Contribution to Project:	Ms. Ortiz analyzed the DNA methylation landscape at the single cell level for primary tumor, CTCs, and metastasis (Gaol 1A).

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

Nothing to report

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: N/A

QUAD CHARTS: N/A

9. APPENDICES: