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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 aim 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 second year of this funding award, and the COVID-19 pandemic still had a significant negative impact on our research activities. Despite the challenges, we still made progress in analyzing CTC heterogeneity in morphology and transcriptomes at the single cell level in our existing patient-derived CTC lines, collection of the spatial transcriptomic profiling samples for PDX tissues, as well as investigation of the mechanisms of hypoxia-mediated inhibition of CTC intrinsic interferon and antigen presentation signals.

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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?

This past year is the third year of this funding award. Due to the transition from USC to University of Maryland, Baltimore, our research activities got delayed to certain degree. However, we still made significant progress in all the major tasks outlined below.

Specific Aim 1: To understand the molecular and physical properties of metastasis-initiating CTCs.

Major Task 1: To characterize metastasis-initiating CTC clones.

Since none of the samples we have tested in the previous year showed CTC growth *ev vivo,* we slowed down the CTC collection in the past year and is waiting for a new CTC equipment to arrive soon with the hope that new technology maybe more gentle in isolation for this *ex vivo* culture purpose.

In the previously report, we have shown that we analyzed the CTC morphology in the established CTC lines and found three major groups of CTCs based on morphological features, in which we termed “Small cell, Large mitochondria, Rough membrane” (SLR), “Small cell, Small mitochondria, Rough cell membrane” (SSR), and “Large cell, Small mitochondria and Smooth cell membrane” (LSS). In the past year, we have performed more functional analyses of these CTC groups.

<u>SLR CTCs divide slowly *in vitro* but have higher tumorigenic capabilities *in vivo*</u>

To test whether the three morphometrically distinct groups have different functions, we will need a larger number of cells to represent each group. Therefore, we optimized and validated a FACS strategy to sort cells that represent the three microscopically defined morphometric groups. We mainly focused on the cell size and Mito/Cell ratio as other features are hard to quantify using FACS. The first gating step based on Cell-Tracker Green sorted the largest (large) and the smallest (small) 10% of the whole cell population. The second gating step based on Mito-Tracker DeepRed sorted the "small" fraction into low (lowest 30 to 40%) and high (highest 10%) mitochondria, resulting in a total of three fractions: "Small/Low", "Small/High" and "Large". Microscopic investigations of cells from these fractions confirmed that the "Small/High" and the "Small/Low" cells were smaller in size than that of the "Large" group. Moreover, the "Small/High" group and the "Large" group showed significantly higher Mito/Cell size ratio than that of the "Small/Low" group. These analyses showed that the FACS sorted fractions ("Small/High", "Small/Low" and "Large") mimic the SLR, SSR, and LSS, respectively (Figure 1A). Therefore, these terms were used in this manuscript to refer to either microscopically defined groups or their respective FACS sorted cell groups.

We hypothesized that the SLR cells may have a higher metastatic ability. The small size may allow them to pass through narrow blood capillaries and the high mitochondrial content may support for energy dependent metastatic steps. To test this hypothesis, we monitored morphometric features during CTC proliferation *in vitro* and tested their tumorgenicity *in vivo*.

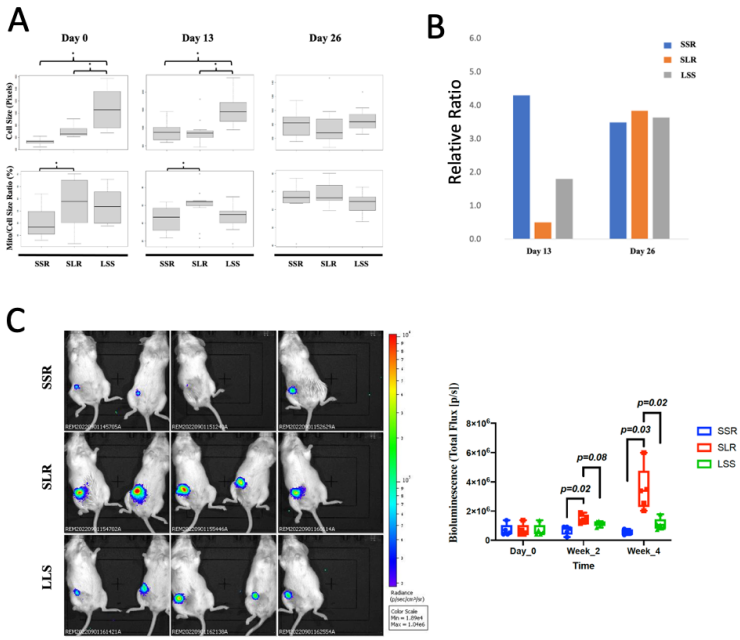

Figure 1. SLR CTCs divide slowly *in vitro* but have higher tumorigenic capabilities *in vivo*. A) Microscopically measured cell size and Mito/Cell size ratio of FACS sorted cells at day 0 of sorting and at days 13 and 26 days of *ex vivo* culture. Cell size was extracted from Cell-Tracker green 2D images using CellProfiler and presented in pixels as described in the methods. Mito/Cell size ratio (%) was calculated by dividing mitochondria size (pixels), as extracted from Mito-Tracker DeepRed 2D images, by cell size then multiplying by 100. B) Cell proliferation rate of CTCs clusters over 26 days. Graph shows the total cell numbers at one time point divided by that at the previous time point. C) *In vivo* imaging of NSG mice which are transplanted with BRx68_luc CTCs' clusters into the 4th MFP at week 4 of transplantation. D) Quantification of bioluminescence signals at different time points. Each dot represents the total Flux (p/s) from one mouse at a given time point. Mice were imaged at day 0, and weeks 2 and 4 post implantation.

When cultured *in vitro*, the SLR cells showed a reduction in cell number from 1000 to 500 cells during the first two weeks, whereas the SSR cells showed highest proliferation rate followed by the LSS cells (Figure 1B). Microscopic imaging at day 13 showed that the morphometric characteristics of the three groups were preserved. Both SLR and SSR cells showed small cell size compared with the LSS group. Moreover, the Mito/Cell size ratio was significantly higher in the SLR than that of the SSR cells (Figure 1A). By day 26, SLR cell proliferation rate increased from day 13 to what is comparable to other groups (Figure 1B). The boost

in SLR cell proliferation seems to have been caused by the emergence of other cell morphologies. Microscopic imaging at day 26 showed that SLR cells are no longer smaller than that of LSS cells and no longer having larger mitochondrial content compared with the SSR cells (Figure 1A).

To test the tumorigenic capability potentially associated with cells with morphometric differences, we used BRx68 cells which are transduced with lentivirus carrying luciferase (BRx68_luc) to allow *in vivo* monitoring of tumor growth. A FACS protocol was optimized to sort BRx68_luc cells based on their size and mitochondrial content and cells from 3 groups were sorted and 200 cells per mouse were injected into the mammary fat pads (MFP) of the female NSG mice. Interestingly, the SLR group showed the highest tumorigenic activity compared to the LSS and the SSR groups (Figure 1C). At week 2 post implantation, the SLR tumors showed a significantly higher bioluminescence than that of the SSR group. There was no significant difference in the bioluminescence between SLR and LSS. By week 4, SLR tumors were significantly larger than that of the two other groups (Figure 1D). This data showed that despite a slower proliferation *in vitro*, the SLR cells have a higher tumorigenic capacity *in vivo* compared to the two other types.

Therefore, based on these data, we are performing some RNA-seq analysis of the different morphological groups for potential underlying molecular changes that are associated with the morphology.

Major Task 2: To analyze and target physical properties of CTCs.

In the previous years, we have reported the identification of a truncated *KRT81* (tKRT81) that is upregulated in metastatic derivatives of CTCs compared to the isogenic, parental CTC lines and is associated with lung metastasis free survival. Further in vitro characterization showed that the truncated KRT81 could bind to KRT18 and disrupt the intermediate filament, leading to softer cells that are more adherent to collagen substrate. Now we have performed in vivo experiments to see whether high expression of tKRT81 increases the metastatic potential.

<u>Expression of tKRT81 promotes in vivo lung metastasis</u>

We evaluated the effect of tKRT81 expression on tumorigenesis and metastasis in immunodeficient NSG mice. Primary tumors generated by mammary fat pad orthotopic injection of 2.5x105 LuM1 cells with tKRT81 expression control (shSCR) or knocked down (shtKRT81) showed no significant difference in size and growth over the 26-week period (Figure 2A). To test for lung recolonization ability, the same cells were injected by lateral tail vein, and mice were monitored by bioluminescent imaging for a period of 32 weeks. At the experimental endpoint, although there was no statistical significance in whole body bioluminescent signal (Figure 2B), there was a significant difference in lung tumor burden when imaged ex vivo (Figure 2C). Similar lateral tail vein injections with the MDA-MB-361 tKRT81-expressing control (NT), tKRT81 knockdown (sg10), and rescue (tKRT81) cell lines showed a significant difference between the rescue and knockdown groups both by whole body imaging (Figure 2D) and in ex vivo measured lung tumor burden (Figure 2E). Although we did not detect any differences between the tKRT81-expressing control and tKRT81 knockdown groups, this may be due to differences in total protein expression levels. The rescue cell line has a 2-fold higher abundance of tKRT81 than the endogenous levels present in the control cell line (Figure 2F), and therefore, an in vivo phenotype may become more evident if the experiment is carried out for a longer period of time.

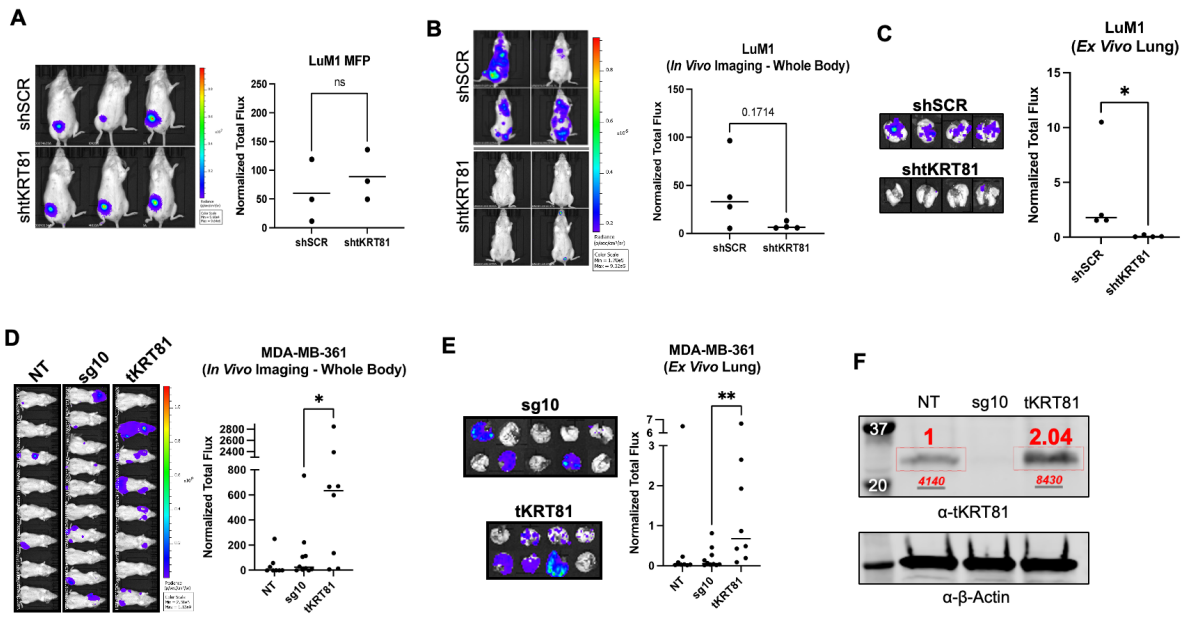

Figure 2. High level of tKRT81 promote lung metastasis in vivo.
(A) Bioluminescence imaging (left) and quantification (right) of orthotopic tumor growth of LuM1 control (shSCR) and tKRT81 knockdown (shtKRT81) cells injected into mammary fat pad of NSG mice. **(B)** Whole body bioluminescence imaging (left) and quantification (right) of NSG mice with tail vein injection of LuM1 control (shSCR) and tKRT81 knockdown (shtKRT81) cells. **(C)** *Ex vivo* bioluminescent lung imaging (left) and quantification (right) of mice 32 weeks after tail vein injection of LuM1 control (shSCR) and tKRT81 knockdown (shtKRT81) cells. *p=0.0143, significance is calculated by one-tailed Mann-Whitney t-test. **(D)** Whole body bioluminescence imaging (left) and quantification (right) of NSG mice with tail vein injection of MDA-MB-361 control (NT), tKRT81 knockdown (sg10), and tKRT81 overexpression rescue (tKRT81) cells. *p<0.05, significance is calculated by unpaired t-test. **(E)** *Ex vivo* bioluminescent lung imaging (left) and quantification (right) of mice 11 weeks after tail vein injection of MDA-MB-361 control (NT) and tKRT81 knockdown (sg10) and overexpression rescue (tKRT81) cells. **p=0.0031, significance is calculated by one-tailed Mann-Whitney t-test. **(F)** Immunoblotting with antibody against KRT81 and β-Actin for cells used in (D). Red numbers were the normalized quantification of tKRT81.

tKRT81 promotes transendothelial cell migration in vivo.

To elucidate the potential molecular mechanism that can explain the enhanced metastases by tKRT81, we evaluated the migration in vitro and transendothelial cell migration in vivo. There was no difference observed with in vitro transwell migration (Figure 3A) or invasion (Figure 3B); however, a significant decrease with in vivo lung transendothelial migration was observed in MDA-MB-361 sg10 cells lacking tKRT81 using a highly sensitive luciferase activity assay on whole lung lysates harvested 24 hours after tail vein injection (Figure 3C). Although the tKRT81 rescue cells (tKRT81) had significantly higher luciferase activity compared to tKRT81 knockdown cells (sg10), a full reversal to the metastatic phenotype of the control cells (NT) was not observed and the value of luciferase activity in the tKRT81 expressing cells was below the limit of detection (LOD). To examine why this may be the case, we visualized the tKRT81-DDK overexpression in the rescue cells using immunofluorescence against the DDK tag and found that the overexpression of DDK is highly heterogeneous (Figure 3D). Given that lungs are harvested only 24 hours after tail vein injection for

this assay, heterogeneity of overexpression in the tKRT81 rescue cells may result in a lower number of cells entering the lungs in that short time frame compared to the unaltered control cells.

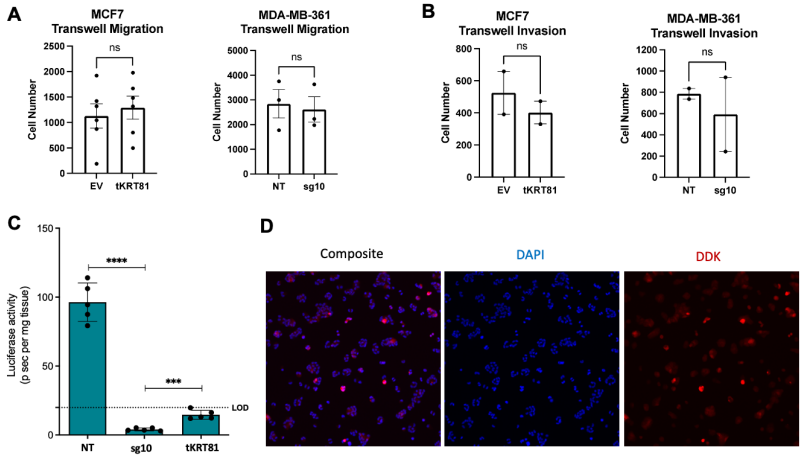

Figure 3. tKRT81 does not influence migration and invasion but promotes transendothelial cell migration.
(A-B) Bar graphs showing number of cells on the bottom of the transwell membrane in transwell migration **(A)** for MCF7 control (EV) and tKRT81 overexpression (tKRT81) cells or MDA-MB-361 control (NT) and tKRT81 knockdown (sg10) cells and transwell invasion **(B)** assay for the same cell lines. Mean ± SEM. **(C)** Graph showing the number of transmigrated control (NT), tKR81 knockdown (sg10), or tKRT81 overexpression (DIMB) MBA-MD-361 cells in mouse lung. *Ex vivo* luciferase activity was quantified from lysates of whole mouse lung extracted 24 hours after intracardiac injection. n = 5, ***p<0.005, ****p<0.0001. Signficance was calculated by two-tailed unpaired *t* test, mean ± SEM. **(D)** Representative images demonstrating the heterogeneity of tKRT81-DDK expression in the MDA-MB-361 tKR81-DDK overexpressing rescue cell line. An anti-DDK was used to visualize DDK-tag expression. All images taken at 4x magnification.

Major Task 3: To profile micrometastasis and dormant DTCs.

Previously, we have reported that using a patient-derived xenograft (PDX) model derived from tumors of an ER+ breast cancer, we picked tumor cells from the center or edge of the primary tumor, metastatic tumors in the liver, lung, and spleen, and CTCs from the same mouse and performed single cell RNA-seq by SMARTseq protocol. We have now finished sequencing for more than 300 samples from 2 mice generated using this PDX model. Data analysis is still ongoing.

Specific Aim 2: To understand the properties of metastasis-supporting niches.

Major Task 1: To analyze the ECM influence on CTCs

Previously, we have reported that in collaboration with Dr. Shelly Peyton from U. Massachusetts Amherst, we have analyzed the ECM effects on CTCs. However, in the last year, Dr. Peyton's lab has met some technical problem in generating the ECM-mimicking hydrogens that need to use. So we are waiting for them to resolve the issue in order to move forward with this sub task.

Major Task 2: To analyze the tumor-stromal cell interaction.

In the previous report, we have shown the single cell multiomics analysis of brain metastases from patients. We have performed analyses to investigate the tumor-stromal cell interactions.

<u>CellChat analysis of the brain metastasis single cell data identified tumor-stromal cell interactions.</u>

We used the CellChat analysis to predict the potential interaction of tumor cells and stromal cells. CellChat is an analysis using the known ligand-receptor pairs and detect the expression levels in the cell types to predict the potential interaction. As shown in Figure 4, many predictions have been made between tumor cells (BrMTC) and other stromal cells, and generated many interesting hypotheses that worth testing as followup studies (Figure 4).

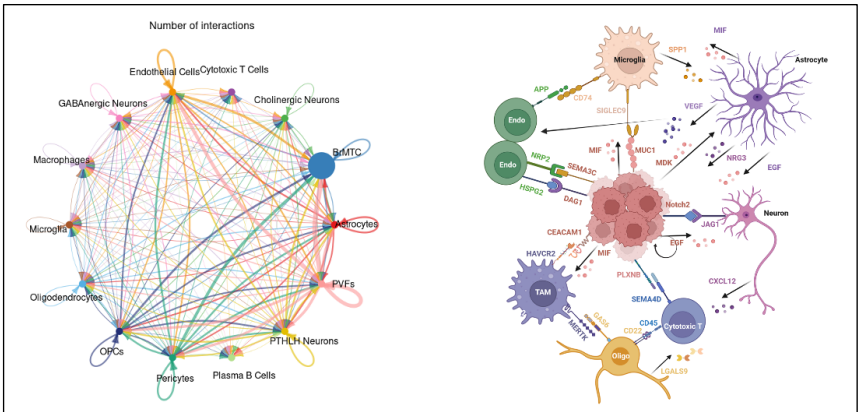

Figure 4. Predicted tumor-stromal cell interactions. Graph on the left showed the predicted interactions between tumor cells and stromal cells, and graph on the right listed the top predicted interations, based on ligand-receptor pair expression in the single cell data using CellChat. Graph on the right was generated using Biorender.

<u>Transcriptional changes of brain metastasis associated stromal cells.</u>

In collaboration with Dr. Nadejda Tsankova lab from Mt. Sinai, we obtained the single nuclei RNA-seq data of the brain autopsies of 3 normal individuals (de-identified). By comparing to the normal stromal cells, we can identify the brain metastasis associated transcriptional changes in the stromal cells. As shown in Figure 5, we can detect many differentially expressed genes in brain metastasis associated microglia compared to the normal microglia, and those changes are the same when compared to the data from Allen Brain Atlas. Those upregulated genes are enriched in pathways such as CLEAR signaling pathway, iNOS signaling, Interferon signaling, and downregulated genes are enriched in pathways related with Calcium signaling, PD-1, PD-L1 cancer immunotherapy pathway. We are currently in the process of analyzing other stromal cell types in a similar way.

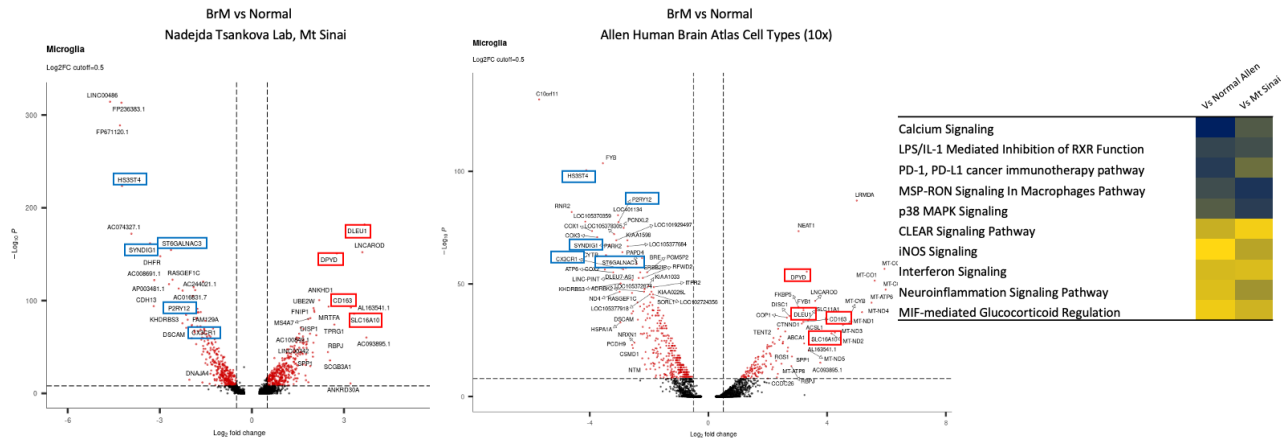

Figure 5. Differentially expressed genes and pathways in brain metastasis (BrM) associated microglia compared to normal microglia. Volcano plots of BrM associated microglia with microglia of 3 autopsy samples (left), Allen human brain atlas (middle), and enriched downregulated and upregulated signaling pathways (right).

Major Task 3: To profile metabolic changes of the metastatic niches.

RNA-seq identified lipid metabolism as a metabolic pathway enriched in metastatic tumors.

Recent data from our laboratory suggests that CTCs may present with diverse metabolic states that potentially contribute to its survival in circulation or the ability to adapt to various organ microenvironments. Our RNA-sequencing analysis of these metastatic CTCs identified organotropism-enriched pathways, among which several are metabolic pathways. One of the most differentially regulated metabolic pathways across multiple metastatic sites in our RNA-sequencing analysis is lipid metabolism (Figure 6). Since lipid metabolism involves both fatty acid synthesis and FAO, we performed initial evaluation on gene expression levels for key enzymes in fatty acid synthesis, but the result showed no meaningful patterns. In contrast, many genes involved in FAO showed elevated expression levels in lung and brain metastasis. So, we focused on FAO.

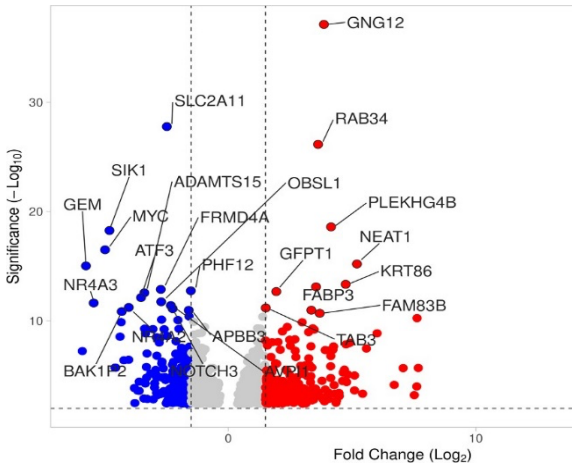

Figure 6. Volcano plot of differentially regulated genes indicated in patient CTC-derived lung metastases. Significantly altered genes in lung metastatic CTCs when compared to the parental CTC cell line from which they were derived. Several genes identified as significant are involved in fatty acid synthesis (FAS) and fatty acid oxidation (FAO).

Detachment from substrate led to increased PPARβ/δ level and cytoplasm-to-nucleus translocation, downstream FAO gene expression, and histone H3 acetylation.

High levels of FAO has been previously associated with a poor clinical prognosis in breast cancer patients. Nuclear receptor superfamily members, peroxisome proliferator-activated receptors (PPARs), are ligand-activated transcription factors that are central regulators in FAO. The three PPAR isoforms, PPARα, PPARβ/δ, and PPARγ control gene expression by binding to specific PPAR response elements (PPREs). Each isoform varies in their ligands, expression patterns, binding partners, and function depending on their tissue-specific distribution. We cultured a well-studied metastatic triple negative breast cancer (TNBC) cell line, MDA-MB-231 cells, under adherent and suspension culture conditions, to represent attached tumor cells and detached cells mimicking CTC escape into the circulatory system. We then performed immunoblot analysis for all three PPAR isoforms to determine if there were any changes in protein expression. Both PPARα and PPARγ showed decreases in expression in suspension conditions,

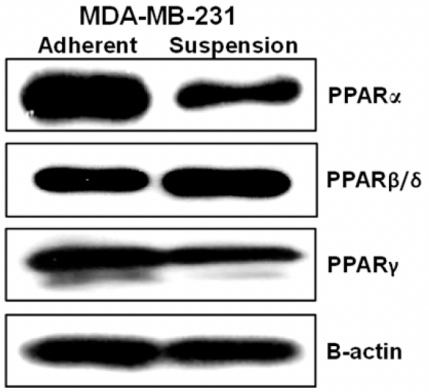

Figure 7: PPAR isoform expression in adherent and suspension MDA-MB-231 cells. Increased protein expression of PPARβ/δ in suspension MDA-MB-231 cells compared to adherent cells. PPARα and PPARγ isoform expression is decreased in suspension conditions.

whereas PPARβ/δ was increased (Figure 7). We then performed nuclear-cytoplasmic fractionation immunoblotting for the attached and detached conditions. Intriguingly, PPARβ/δ showed an obvious translocation into nucleus upon detachment, in contrast to the similar distribution of PPARα and PPARγ no matter the growth condition (Figure 8).

These data are very exciting for us to follow upon, since there are not sufficient understanding of the metabolic changes associated with CTCs compared to attached primary tumor cells. We will focus on understanding the impact of PPARβ/δ on the survival of CTCs in suspension and the molecular mechanisms linking lipid metabolism with the CTC biology.

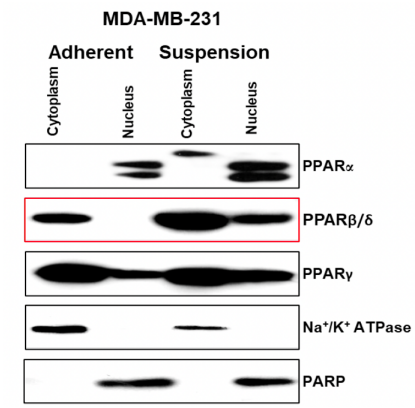

Figure 8: PPAR expression in subcellular fractions of adherent and suspension MDA-MB- 231 cells. Growth of MDA-MB-231 cells in suspension conditions alters the cellular distribution of PPARβ/δ to the nucleus when compared to adherent cells where it is solely expressed in the cytosol.

Specific Aim 3: Target TME-mediated epigenetic memory in CTCs

Major Task 1: To investigate hypoxia-mediated immune evasion

Since previously we have reported that hypoxia downregulate tumor intrinsic interferon (IFN) signal and antigen presentation (AP) pathways and such effect seems to last longer than the hypoxic exposure that we call hypoxic memory. We also have shown that murine mammary tumor cell line NT2.5 cells show similar trend as MCF7 cells in terms of downregulation IFN/AP in hypoxia and with hypoxic memory. To evaluate the effect

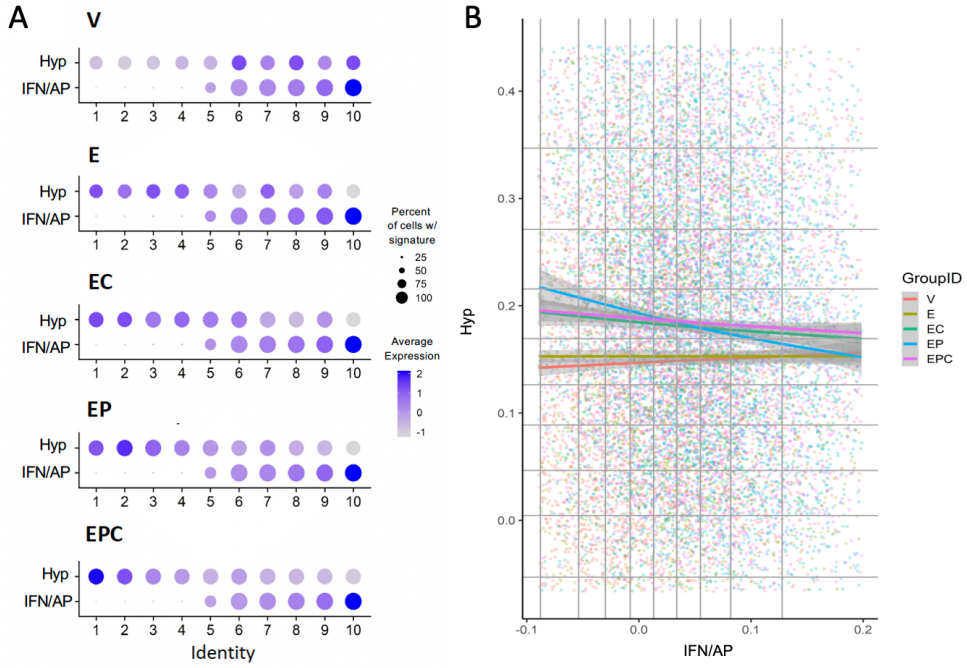

Figure 9. scRNAseq analysis of hypoxia and IFN/AP scores in syngeneic mammary tumors. (A) scRNAseq analysis of hypoxia and IFN/AP scores in tumor cells in the syngeneic mammary tumors formed by NT2.5 cells, treated with vehicle (V), Entinostat (E), Entinostat combined with anti-CTLA4 antibody (EC), with anti-PD1 antibody (EP), or with both (EPC). Decile plot of hypoxia and IFN/AP scores for cell population ranked based on IFN/AP scores. **(B)** Dot plot showing IFN/AP (x-axis) and hypoxia (y-axis) scores for tumor cells (within 5-95 percentiles) for all treatment conditions.

in vivo, we performed scRNAseq analysis of the NT2.5 cells in syngeneic tumors treated with vehicle, Entinostat alone, or in combination with anti-CTLA4, anti-PD1, or both(Christmas et al., 2018). A total of 20 tumors (4 from each group) were processed by 10x Chromium platform after 3 weeks of treatment, but before a significant tumor shrinkage. The data was generated by our collaborator Dr. Evanthia Rossous Torres at USC. Decile plots of the tumor cells ranked by IFN/AP score showed a clear inverse correlation of hypoxia and IFN/AP signatures after Entinostat treatment, and the effect became even more obvious when combined with the immunotherapies (Figure 9A). Compared with vehicle condition, Entinostat treatment shifted these 2 features of the tumor cells. The most obvious change seemed to occur in the population of the vehicle condition with double negative scores for both hypoxia and IFN/AP, which may contain tumor cells that have left the hypoxic region but retain a hypoxic memory of IFN/AP downregulation. It is possible that Entinostat removed the hypoxic memory and upregulated the IFN/AP signals for tumor cells in the normoxic region (negative for hypoxia). This corroborates the above *in vitro* finding of Entinostat erasing the hypoxic memory of IFN target suppression, but not so significantly in hypoxic conditions. The combination of Entinostat with checkpoint blockades led to an overall increased hypoxia score in the tumors (Figure 9B), suggesting more effective elimination of cells in normoxia.

Major Task 2: To investigate hypoxic long-term effect in metastasis

Major Task 3: To investigate mechanisms of hypoxia-mediated epigenetic memory

In order to determine the hypoxic long-term effect in transcription and epigenetic regulation, we performed the RNA-seq and CUT&TAG for a list of histone marks for MCF7 and MDA-MB-231 cells cultured in normoxia, hypoxia (4% or 1%), for either 3 days or 14 days, and reoxygenated for 3 days (Figure 10). As illustrated in Figure 10, we have performed the experiments and submitted the samples for sequencing. We have just received the results back and still in the middle of data analysis. We believe with this unbiased analysis, we will gain a grand view of the long-term effect of hypoxia exposure and associated signaling that promote metastasis.

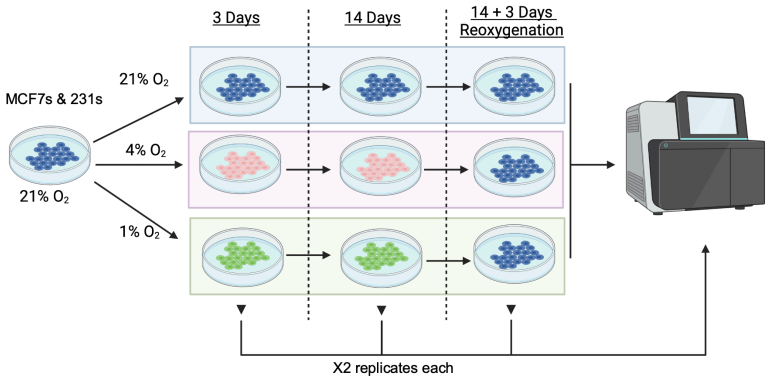

Figure 10. Illustration of the experimental setup.

Other achievements

Nothing to report

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

Nothing to report

How were the results disseminated to communities of interest?

I have discussed some of the results presented here to two breast cancer patient advocates.

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

We recently moved from USC to University of Maryland, Baltimore. We plan to work with clinicians at UMB to recruit new blood samples from metastatic breast cancer patients to isolate, analyze CTCs and aim to establish more CTC lines. We will have to obtain a new CTC isolation system from TellBio which can efficiently deplete the contaminating leukocytes since we couldn't take the TellBio system from USC. We are hoping that this system will allow us to establish more CTC lines and potentially single CTC clones from metastatic breast cancer patient samples. We will also focus on pairing the morphological and transcriptomic analysis at the single CTC level and to determine whether there are certain signaling pathways being reflected as unique morphological features. We plan to sort the single CTCs with the distinct morphological features as shown in Figure 1 to perform single cell RNA-seq analysis. We plan to continue the spatial transcriptomic analysis of the PDX models to identify transcriptional changes associated with various steps of metastatic cascade, using samples listed in Figure 5. We will focus on data analysis and validation of candidates in the upcoming year. For the physical property analysis, we have submitted the manuscript related with tKRT81 and it is currently under review. 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 as in Figure 4, focusing on the ligand-receptor pairs between tumor and stromal cells, as well as the stromal cell changes when compared to normal stromal cells. We will start to perform validation studies using IHC or IF staining, or spatial transcriptomic analysis on the metastatic tissue samples. Moreover, we continue to investigate the epigenetic mechanism and impact of hypoxia-mediated long term effect, with the focus on analyzing the sequencing data that we obtained with experiment stated as in Figure 10. We will identify what other signaling pathways and underlying mechanisms occurring together with 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

We recently moved from USC to UMB, which has delayed to some extent the progress of some part of the project. Together with the significant impact of COVID pandemic in the first 2 years of the project, 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

Nothing to report

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

Has to change to new institution, waiting for IRB to be approved.

Significant changes in use or care of vertebrate animals

Has to change to new institution, waiting for IACUC to be approved.

Significant changes in use of biohazards and/or select agents

Has to change to new institution, waiting for IBC to be approved.

6. PRODUCTS:

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

Journal publications.

Sidiropoulos D, Rafie C, Jang J, Castanon S, Baugh A, Gonzalez E, Christmas B, Narumi V, Davis-Marcisak E, Sharma G, Bigelow E, Vaghasia A, Gupta A, Skaist A, Considine M, Wheelan S, Ganesan SK, **Yu M**, Yegnasubramanian S, Stearns V, Connolly R, Gaykalova D, Kagohara L, Jaffee E, Fertig E, and Roussos Torres E. Entinostat decreases immune

suppression to promote an anti-tumor response within a HER2+ breast tumor microenvironment. ***Cancer Immunology Research*****,** 2022

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

Kang DS, Moriarty A, Oh JM, Begum HM, Shen K, **Yu M**. Biophysical properties and isolation of circulating tumor cells. (Book chapter) ***Engineering and Physical Approaches to Cancer***. Accepted.

Begum HM, Oh JM, Kang DS, **Yu M**, Shen K. Physical regulations of metabolism and cell interactions in tumor microenvironments. (Book chapter) ***Engineering and Physical Approaches to Cancer***. Accepted.

Other publications, conference papers and presentations.

2022	Lecture	University of Maryland Medical School, Baltimore, MD, Circulating tumor cells inform mechanisms of breast cancer metastasis
2023	Symposium speaker	The first brain metastasis symposium, MSKCC, (virtual), Single cell analysis of brain metastasis
2023	Lecture	NCI, Circulating tumor cells inform mechanism of breast cancer metastasis

- **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 (up till 1/16/2023)?

Name:	Min Yu, PhD
Project Role:	PI
Researcher Identifier:	ORCID ID: 0000-0002-3969-8720

Nearest person month worked: 3.6 calendar months
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 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: 12 calendar months
Contribution to Project: Dr. Saleh is the main person who developed the protocol for staining, scanning and picking live CTCs on RareCyte. He will be the core user for the RareCyte system. In this proposal, he will carry out the experiment to analyze patient CTCs using RareCyte and generate single CTC clones as proposed in Goal 1. In addition, he will also carry out the experiments proposed in Goal 1C on micrometastasis and dormant DTC analysis.

Name: Remi Klotz, PhD
Project Role: Research Associate
Researcher Identifier: N/A

Nearest person month worked:	1.2
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:	Diane Kang
Project Role:	Graduate Student
Researcher Identifier:	N/A
Nearest person month worked:	5.2 calendar months
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:	Postdoc
Researcher Identifier:	N/A
Nearest person month worked:	2 calendar months
Contribution to Project:	Dr. Ortiz analyzed the DNA methylation landscape at the single cell level for primary tumor, CTCs, and metastasis (Goal 1A).
Name:	Irving Garcia
Project Role:	Lab Technician
Researcher Identifier:	N/A
Nearest person month worked:	1 calendar months
Contribution to Project:	Mr. Garcia provided technical assistant for Dr. Saleh and Dr. Ortiz for their research in this project, including maintaining the animal colonies, maintaining the consistency of the reagents and protocols.
Name:	Sarai Plummer
Project Role:	Lab Technician
Researcher Identifier:	N/A
Nearest person month worked:	5.5 calendar months
Contribution to Project:	Ms. Plummer provided assistance to Dr. Saleh on the single cell RNA-seq analysis of the PDX models for Goal 1C on micrometastasis and dormant DTC analysis.
Name:	Amal Thomas
Project Role:	Graduate Student
Researcher Identifier:	N/A
Nearest person month worked:	1.1 calendar months
Contribution to Project:	Ms. Thomas performed data analysis and interpretation under the supervision of Dr. Smith.

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

The following previously active grants have been closed:

1R01CA241137-01S1 (Mumenthaler) 8/1/2021-7/30/2022 0.36 CM
NIH/NCI (to co-I Yu)
A microengineered colon cancer-chip designed to investigate tumor-stromal interactions driving cancer progression
This is a supplement grant and the major goal of this supplement is combining a biomimetic OOC model with a mathematical model to test our hypothesis that peristalsis-like motion promotes CRC tumor cell intravasation and phenotypic switching that results in increased metastatic potential.
Role: co-I
Overlap: None

MHI for Research of Engineering Medicine for Cancer (Yu, Zhang, Kang) 8/1/2021-7/30/2022
USC Ming Hsieh Institute
Developing anti-SEMA4D antibody drug conjugate to prevent breast cancer to brain metastasis
The goal of this project is to evaluate the potential of anti-SEMA4D antibody drug conjugate as a prophylactic therapy for brain metastasis
Role: co-PI
Overlap: None

Summary of In-Kind Contribution:
Visiting Scholar, Dr. Yilin Yi, who conducts research activities in the Yu Lab. Salary supported by Peking University Cancer Hospital.
Status of Support: Active
Primary Place of Performance: University of Southern California, Los Angeles
Project/Proposal Start and End Date (MM/YYYY) (if available): 10/01/2019 – 11/30/2021
Estimated Dollar Value of In-Kind Information:

The following grants are new active grants:

None

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: N/A

QUAD CHARTS: N/A

9. APPENDICES: