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TITLE: Mitochondrial horizontal transfer in breast cancer cells

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CONTRACTING ORGANIZATION: University of Utah

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Cancer cells constantly interact with surrounding cells in the tumor. The communication between cancer cells and surrounding cells is often the reason behind the failure of cancer therapeutics. We seek to understand this communication in an effort to overcome these barriers. Macrophages are a component of the immune system. Their best known function is to "seek and destroy" foreign particles. However, in the tumor environment, macrophages have a very different role – macrophages can facilitate steps of metastasis by communicating with cancer cells and helping cancer cells leave the tumor, enter the blood stream, and grow at the metastatic site. We discovered that macrophages transfer mitochondria to triple negative breast cancer cells, regulating cancer cell proliferation. We polarized macrophages to an M2-like state and found increased mitochondrial transfer to cancer cells. Furthermore, increasing and decreasing mitochondrial fragmentation in macrophages by overexpressing and inhibiting a key mitochondrial fission machinery player, DRP-1, led to increased and decreased mitochondrial transfer, respectively. We are now optimizing conditions to assess macrophage mitochondrial transfer to patient-derived cells, and are performing experiment to mechanistically understand how macrophage mitochondrial transfer leads to increased breast cancer cell proliferation.

15. SUBJECT TERMS

Triple negative breast cancer, metastasis, mitochondrial transfer, patient-derived cells, reactive oxygen species

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1. INTRODUCTION:

There is growing evidence that macrophages can modulate cell behavior via unconventional cell contact-mediated communication in the contexts of development and homeostasis. We have recently extended these paradigms by discovering that macrophages can also engage in unconventional cell contact-mediated communication with tumor cells within the tumor microenvironment, and that these interactions contribute to metastasis. Macrophages horizontally transfer mitochondria to triple negative breast cancer cells. We aim to determine how mitochondrial transfer is regulated, and how transferred mitochondria affect breast cancer cell behavior. Our project will define how stromal cell organelle contributions alter cancer cell behavior, and will provide a basis for developing future immunotherapies that limit metastasis.

2. KEYWORDS:

Triple negative breast cancer, metastasis, mitochondrial transfer, patient-derived cells, reactive oxygen species

3. ACCOMPLISHMENTS:

What were the major goals of the project?

	Proposed Timeline (months)	Completion Date/Progress
Specific Aim 1: Manipulating macrophage mitochondrial dynamics and transfer to breast cancer cells		
Major Task 1: Manipulate macrophage mitochondria morphology to determine effects on mitochondrial transfer		
Establish effective shRNA knockdown strategies in primary macrophages for use in subsequent aims	1-3	December 2020 - DRP1 shRNA in macrophages, confirmed with qRT-PCR
Establish quantitative pipeline with analysis software to assess mitochondrial morphology	3-5	March 2021 - implemented and optimized Mitochondrial Network Analysis (MiNA) software to quantify mitochondrial morphology
Determine whether changes in macrophage mitochondria morphology affect mitochondrial transfer with flow cytometry	5-7	May 2021 - determined that DRP1 shRNA macrophages exhibit decreased mitochondrial transfer to breast cancer cells
Milestone: Ability to inhibit mitochondrial transfer to MDA-MB-231 breast cancer cells	7	May 2021 - Achieved

Major Task 2: Assessing how macrophage polarization affects mitochondrial transfer to MDA-MB-231 and patient-derived cells		
Establish and validate efficient macrophage polarization methods for use in subsequent aims	7-9	Feb 2021 - confirmation of macrophage polarization using single markers; next step involves additional markers
Determine effects of macrophage polarization on mitochondrial transfer with flow cytometry	9-12	April 2021 - determined that M2-like polarized macrophages exhibit increased mitochondrial transfer to breast cancer cells
Establish coculturing methods for macrophages with patient derived xenograft organoids in 2D & 3D	11-15	on-going - established mitochondrial transfer to one line of patient-derived xenograft organoid cells in 3D; currently testing with others in 2D and 3D
Milestone: Macrophage polarization conditions for efficient mitochondrial transfer to patient-derived xenograft organoids	18	on-going

What was accomplished under these goals?

Major Activities – The main activity during this reporting period was research-based – acquisition and analysis of macrophage polarization and mitochondrial morphology, and how these changes affect macrophage mitochondrial transfer to breast cancer cells.

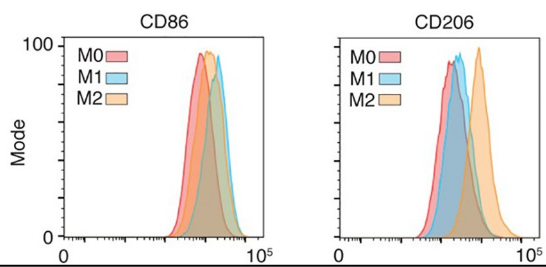

Fig. 1: Macrophage polarization. Macrophages were activated with IFN-γ (M1 activation; left) or IL-4/IL-13 (M2 activation; right) for 48 hours and flow cytometry was used to determine expression of canonical M1 (CD86, left) and M2 (CD206, right) markers. Representative histograms shown.

Specific Objectives – Our objectives were to 1) determine whether macrophage polarization affects mitochondrial transfer to breast cancer cell lines and patient-derived cells, and 2) determine whether macrophage mitochondrial morphology directly affects mitochondrial transfer to breast cancer cells.

Significant Results – In many solid tumors, macrophages are differentiated and function pro-tumorigenically. Therefore, we tested how macrophage polarization status affects mitochondrial transfer. We broadly stimulated macrophages into pro-inflammatory M1-like and pro-tumorigenic M2-like macrophage subtypes. Macrophages were harvested between days 6-7 of differentiation, and IFN-γ (20 ng/mL) for M1-like polarization or IL-4 + IL-13 (20 ng/mL) for M2-like polarization were added to culture media for 48 hours before experiments were conducted. To confirm M1-like and M2-like activation, macrophages were collected and stained for known surface markers for M1 (CD86) and M2 (CD206) activation (Fig. 1).

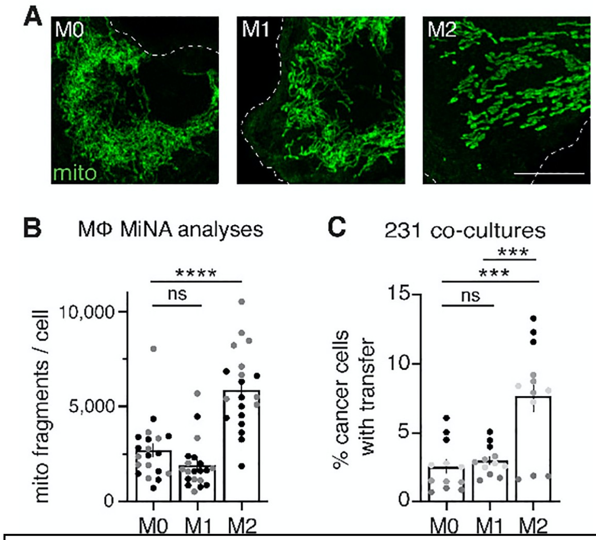

Fig. 2. M2-like macrophage activation increases mitochondrial fragmentation and transfer to breast cancer cells. (A) Representative images of mito-mEm+ macrophages that were non-stimulated (M0, left) or activated to become M1-like (middle) or M2-like (right) for 48 hours. (B) Mitochondrial network analyses (MiNA) were used to determine number of mitochondrial fragments per cell (N=2). (C) Macrophages were co-cultured with mito-RFP 231 cells for 24 hours and transfer was quantified with flow cytometry (N=4).

To quantify mitochondrial morphology in M1-like and M2-like macrophages, we used Mitochondrial Network Analaysis (MiNA). A 'mitochondrial fragment' was defined as a mitochondrion with 0-1 branches, 0 junctions, and a length between 0-2 µm. With this analysis tool, we determined that M2-like macrophages exhibited increased mitochondrial fragmentation compared to M1-like and M0 (unstimulated) macrophages (**Fig. 2A-B**). We then asked whether different macrophage subsets exhibit differences in mitochondrial transfer to breast cancer cells. To answer this question, we performed flow cytometry for macrophage mitochondrial transfer, and found that M2-like macrophages exhibit increased mitochondrial transfer to breast cancer cells (**Fig. 2C**).

These results suggest that smaller mitochondrial fragments would be more readily transferred than larger mitochondrial networks; therefore, we directly manipulated mitochondrial morphology by modulating a key regulator of mitochondrial fission, DRP1. Macrophages treated with DRP1-shRNA and DRP1-mCherry overexpression resulted in hyper-fused and hyper-fragmented mitochondrial networks, respectively (MiNA analysis, **Fig. 3A**). Furthermore macrophages with hyper-fused and hyper-fragmented mitochondrial networks resulted in decreased and increased mitochondrial transfer (**Fig. 3B**). These results suggest that mitochondrial morphology directly contributes to mitochondrial transfer.

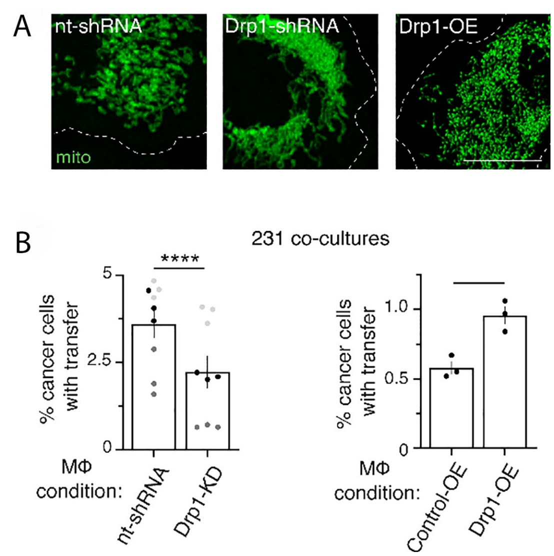

Fig. 3. Increased mitochondrial fragmentation increases transfer. (A)Representative images of mito-mEm (green) macrophages transduced with lentiviruses (upper left corner). (B) Rates of mitochondrial transfer with macrophages with Drp1-knockdown (KD; left) or Drp1-overexpression (OE; right) compared to appropriate controls (N=3).

We have met all of the goals proposed in the research plan for this reporting year.

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

This project has provided extensive research training to Joseph Casalini, Daniel Greiner, Julio Fierro, and Chelsea Kidwell in cancer biology, mitochondrial biology, patient-derived cells, 3D culturing techniques, and data analysis.

All three trainees have also attending a number of virtual conferences over the past year to disseminate their knowledge and train in scientific communication. These conferences include: Metastasis Breast Cancer Research Conference (September 2020), Myeloid Cells and Innate Immunity in Solid Tumors Keystone Meeting (September 2020), and the American Society for Cell Biology (December 2020).

How were the results disseminated to communities of interest?
Members of the lab have presented findings related to this work at the Metastasis Breast Cancer Research Conference (virtual, September 2020), Myeloid Cells and Innate Immunity in Solid Tumors Keystone Meeting (virtual, September 2020), and the American Society for Cell Biology (virtual, December 2020).
We did not participate in in-person outreach this reporting year due to the on-going pandemic.

What do you plan to do during the next reporting period to accomplish the goals?
Our priorities for the next reporting period are as follows:

1. Assess mitochondrial transfer to patient-derived xenograft organoids: We have generated preliminary evidence that suggests macrophages transfer mitochondria to triple negative breast cancer patient-derived xenograft organoids (PDxO), however, we will continue these studies to confirm our preliminary findings, as well as to test mitochondrial transfer with additional PDxO cells. We will also test whether macrophage mitochondrial transfer increases or decreases with PDxO cells derived from a primary tumor versus a metastasis, and determine whether transferred mitochondria are dysfunctional as we have observed with our breast cancer cell lines.
    a. Determine coculturing conditions across 6 PDxO cells (2 patients; for each patient, 1 primary tumor and 2 metastasis)
    b. Optimize conditions for detection of mitochondrial transfer to PDxO with flow cytometry
    c. Optimize conditions for mitochondrial labelling to assess mitochondrial function
2. Manipulate reactive oxygen species generation in cancer cells: We had previously determined that transferred mitochondria accumulate reactive oxygen species (ROS); however, our goal for the next reporting period is to manipulate the ROS generation to test whether ROS is necessary and sufficient for changes in breast cancer cell behavior.
    a. Test a series of ROS quenchers and inducers and monitor ROS levels with dyes
    b. Perform mitochondrial transfer experiments and quench ROS in cancer cells
    c. Assess changes in cancer cell behavior with behavioral assays

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?
Noting 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
Nothing to report.

Changes that had a significant impact on expenditures
We have spent more money on salary than had originally been budgeted during this reporting period due to our post-doctoral fellow, Dr. Chelsea Kidwell's expertise being required on the project. We anticipate that these additional salary expenditures will not continue to be required throughout the duration of the project.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report.

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.

Oral Presentations at Conferences and Other Institutions:

Kidwell CU, Casalini J, Johnson JS, Roh-Johnson M. Horizontal transfer of macrophage mitochondria during metastasis. *Myeloid Cells and Innate Immunity in Solid Tumors Keystone Meeting, virtual*

Kidwell CU, Casalini J, Johnson JS, Roh-Johnson M. Horizontal transfer of macrophage mitochondria in cancer. *Reed College*, virtual.

Kidwell CU, Casalini J, Johnson JS, Roh-Johnson M. Horizontal transfer of macrophage mitochondria during metastasis. *University of Michigan,* Student-invited speaker, virtual

- **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**
  We have generated a number of constructs to visualize mitochondria and mitochondrial function in cells. These tags were generated in lentiviral backbones for efficient transduction of primary macrophages. We plan to make these constructs available on Addgene once the manuscript describing these constructs is accepted.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Minna Roh-Johnson
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	https://orcid.org/0000-0003-3961-4547
Nearest person month worked:	2
Contribution to Project:	Dr. Roh-Johnson has provided overall project development and oversight. She has trained members of the lab and has assisted personnel on experimental design and data interpretation.
Funding Support:	NIH/NCI R01CA247994 (PI); Mary Kay Foundation Innovation Award (PI); NIH/NCI R00CA190836 (PI)

Name:	Chelsea Kidwell
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	https://orcid.org/0000-0003-4269-2503
Nearest person month worked:	6

Contribution to Project: Dr. Kidwell has performed all experiments with patient-derived xenograft organoids, and has optimized all experiments using primary macrophages.
Funding Support: NIH/NCI R00CA190836 Diversity Supplement

Name: Daniel Greiner
Project Role: Graduate Student
Researcher Identifier (e.g. ORCID ID): https://orcid.org/0000-0001-6272-3237
Nearest person month worked: 6
Contribution to Project: Mr. Greiner performed macrophage mitochondrial transfer experiments, and assisted with patient-derived xenograft organoid experiments.
Funding Support: No other direct funding support.

Name: Julio Fierro
Project Role: Graduate Student
Researcher Identifier (e.g. ORCID ID): https://orcid.org/0000-0003-4788-8636
Nearest person month worked: 5
Contribution to Project: Mr. Fierro assisted with bioinformatics analysis of mitochondrial morphology for both macrophage polarization and fission machinery knockdown.
Funding Support: No other direct 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?
Yes, Dr. Roh-Johnson is now supported by NIH grant 1R01CA247994.

What other organizations were involved as partners?
Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: N/A

QUAD CHARTS: N/A

9. APPENDICES:

Award chart attached.

Award Log Number: Award Title

PI: Name, Institution, State
Budget: Total Award Cost
Topic Area: Program Name
Mechanism: Funding Opportunity

Research Area(s): 0406
Award Status: 08/01/20 – 07/31/21

Study Goals:

The goals of this project are 2-fold: 1. To develop a mechanistic understanding of mitochondrial lateral transfer from immune cells to triple negative breast cancer cells to design potential biomarkers; and 2. To generate a strong foundational knowledge of the diverse functions of immune cells in the tumor microenvironment to develop more effective immunotherapies for triple negative breast cancer.

Specific Aims:

Aim 1: Determine how macrophages regulate mitochondrial dynamics and mitochondrial transfer to breast cancer cells.
Aim 2: Determine how transferred mitochondria affect breast cancer cell behavior

Key Accomplishments and Outcomes:

Publications: none to date

Patents: none to date

Funding Obtained:
NIH/National Cancer Institute
R01CA247994-01 (Roh, PI)
02/01/2021 – 01/31/2026