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TITLE: Mitochondrial Horizontal Transfer in Triple-Negative Breast Cancer

PRINCIPAL INVESTIGATOR: Minna Roh-Johnson

CONTRACTING ORGANIZATION: University of Utah

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6. AUTHOR(S)
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E-Mail:

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

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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

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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Unclassified	Unclassified	Unclassified	Unclassified	13	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

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 2: Assessing how macrophage polarization affects mitochondrial transfer to MDA-MB-231 and patient-derived cells		
Assess mitochondrial transfer to patient-derived xenograft organoids in 2D & 3D with flow cytometry	15-18	August 2021 - established mitochondrial transfer to patient-derived xenograft organoid cells in 3D
Milestone Achieved: Determination of macrophage polarization conditions for efficient mitochondrial transfer to patient-derived xenograft organoids	18	August 2021 - Achieved
Specific Aim 2: Determine how transferred macrophage mitochondria affect MDA-MB-231 breast cancer cell behavior.		
Major Task 1 – Manipulate reactive oxygen species generation and redox signaling in breast cancer cells to determine whether reactive oxygen species can act as a signal in MDA-MB-231 cancer cells		
Generate and assess the level of reactive oxygen species with biosensors and dyes at transferred mitochondria in MDA-MB-231 cells	18-20	August 2021 – determined reactive oxygen species accumulation at transferred mitochondria using Grx1 biosensor and Orp biosensor.

Generate and assess the level of reactive oxygen species in primary macrophages with biosensors and dyes before mitochondrial transfer	19-22	On-going – having difficulty expressing the ROS biosensors in primary macrophages
Manipulate reactive oxygen species generation and downstream signaling with chemical inhibitors in MDA-MB-231 cells	21-23	September 2021 – successfully induced reactive oxygen species with mito-KillerRed, but was unable to quench reactive oxygen species in cancer cells.
Assess in MDA-MB-231 cell proliferation when reactive oxygen species levels are manipulated with flow cytometry	23-27	September 2021 – evaluated MDA-MB-231 cell proliferation when reactive oxygen species were induced with flow cytometry
Milestone(s) Achieved: Determine whether reactive oxygen species accumulation at transferred mitochondria serves as a signal regulating in MDA-MB-231 cell proliferation	27	September 2021 – Achieved.
Major Task 2 – Injection of purified macrophage mitochondria into cancer cells to determine whether mitochondria alone can induce changes in MDA-MB-231 breast cancer cell behavior		
Evaluate quality of purified mitochondrial preparations with biochemical approaches	27-29	January 2022 – successfully purified mitochondria from macrophages and evaluated membrane potential
Test whether purified mitochondrial injections induce reactive oxygen species in MDA-MB-231 cells with biosensors	29-32	ongoing
Test whether purified mitochondrial injections induce MDA-MB-231 cell proliferation with flow cytometry	32-36	May 2022 – bath applied purified mitochondrial preparations to MDA-MB-231 cells and induced cancer cell proliferation
Milestone(s) Achieved: Determining whether transferred mitochondria alone can drive metastatic potential of MDA-MB-231 cells	36	ongoing

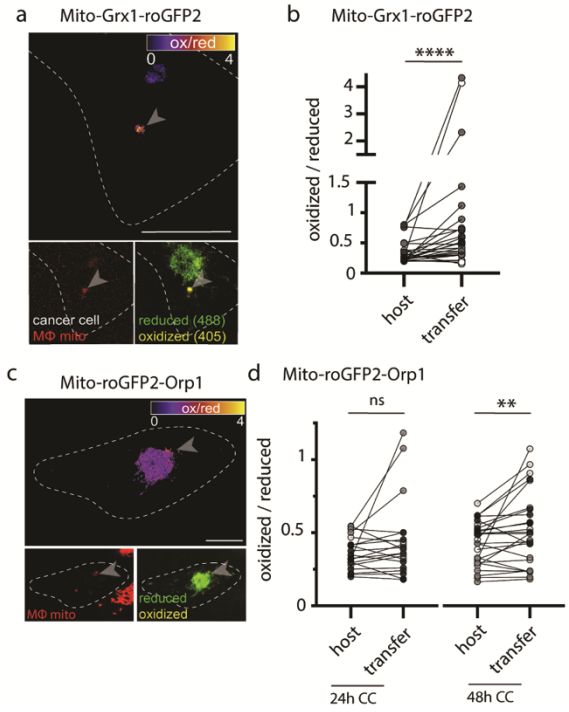
What was accomplished under these goals?

Major Activities – The main activity during this reporting period was research-based – determining how macrophage mitochondrial transfer affects breast cancer cell proliferation, and whether

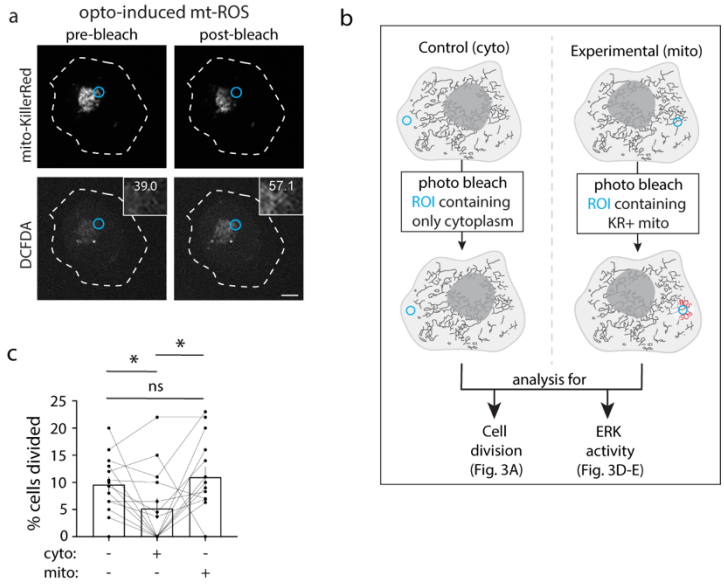
reactive oxygen species at transferred mitochondria acts as a signal in cancer cells regulating proliferation.

Specific Objectives – Our objectives were to 1) determine whether macrophage mitochondria accumulate reactive oxygen species in cancer cells, and 2) determine whether reactive oxygen species accumulation at transferred macrophage mitochondria affects cancer cell proliferation.

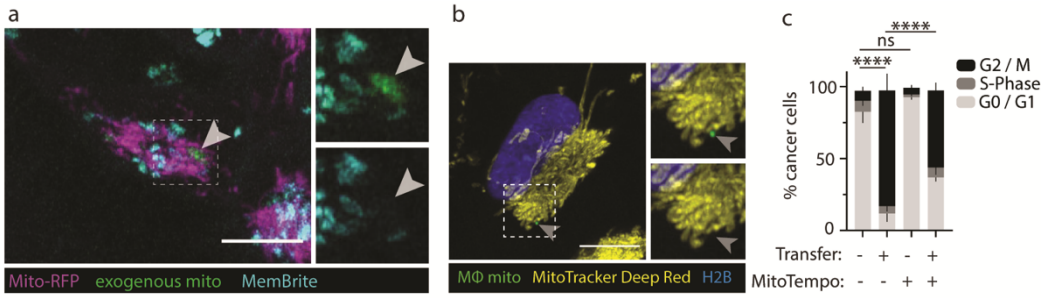
Significant Results – We had already determined that transferred mitochondria lack membrane potential, and thus hypothesized that transferred mitochondria might be dysfunctional. A hallmark of dysfunctional mitochondria is unregulated reactive oxygen species generation. Therefore we tested whether transferred mitochondria accumulate reactive oxygen species in cancer cells. Using a genetically encoded biosensor, mito-Grx1-roGFP2, as a live readout of the mitochondrial glutathione redox state [21], we found that after 24 and 48 hours, significantly higher ratios of oxidized:reduced protein were associated with the transferred mitochondria versus the host network (Fig. 1a,b). These data indicate that transferred macrophage mitochondria in recipient cells are associated with higher levels of oxidized glutathione, suggesting that they are accumulating higher amounts of ROS. Consistent with these results, a second biosensor that is specific for the ROS H_2O_2, mito-roGFP2-Orp1 [22], also reported more oxidation at the transferred mitochondria compared to the host network (Fig. 1c,d) after 48 hours of co-culture. At 24 hours, we observed a similar trend, but no statistically significant difference (Fig. 1d). These results indicate that ROS accumulates at the site of transferred mitochondria in recipient cancer cells, and we next tested whether this ROS accumulation could serve as a signal in recipient cells, regulating cell proliferation.



To test whether ROS accumulation can induce cancer cell proliferation, we stably expressed a mitochondrially localized photosensitizer, mito-KillerRed, which generates ROS when photobleached with 547nm light [23]. As expected, photobleaching mito-KillerRed+ regions of interest induced ROS [24] (Fig. 2a). We then drew mito-KillerRed+ regions of interest that mimicked the size of macrophage mitochondrial transfer to induce local reactive oxygen species, and analyzed the rate of cell division by imaging these cells over 18 hours. We found that cells with induced ROS (by photobleaching mito-KillerRed+ regions) exhibited an increased percentage of dividing cells compared to negative control photobleached cells (mito vs. cyto bleach; Fig. 2b,c). These results indicate that ROS induction alone is sufficient to promote cancer cell proliferation.



To rigorously test the model that transferred macrophage mitochondria accumulate ROS, promoting cancer cell proliferation, we next purified macrophage mitochondria and directly applied these mitochondria to cancer cells and quantified resulting cancer cell proliferative capacity (Fig. 3). We first determined conditions for cancer cells to internalize exogenous macrophage mitochondria at rates similar to *in vitro* mitochondrial transfer conditions – 0.68% ± 0.36% internalization rate, n=3 biological replicates (compared ~1% in *in vitro* mitochondrial transfer conditons). We next determined that purified mitochondria taken up by cancer cells remain distinct, are not encapsulated by membranes after 24 hours (Fig. 3a), and do not exhibit membrane potential (Fig. 3b). We then analyzed proliferative capacity, and found that cancer cells with internalized purified macrophage mitochondria (which are ~1% of the total population) exhibited a significant increase in proliferative cells in the G2/M phase of the cell cycle, compared to sister cells that did not internalize mitochondria (Fig. 3c, comparing black bars in lanes 1&2). These results indicate that transferred mitochondria promote proliferation in cancer cells.



One goal that we have struggled to meet is quenching ROS to test whether cancer cell proliferation is ROS-dependent. We have used a number of different ROS quenchers and ROS dyes to evaluate ROS levels in cancer cells with and without macrophage mitochondria, but have been unable to detect any differences using flow cytometry. We recently purchased a new ROS dye that is marketed as superior sensitivity, and we will be trialing this new reagent in the coming weeks. We were able to perform ROS quenching experiments with our purified macrophage mitochondrial approaches (Figure 3c), but we are still struggling to quench ROS during the in vitro macrophage mitochondrial transfer assays.

What opportunities for training and professional development has the project provided?
This project has provided extensive research training to Daniel Greiner, Julio Fierro, Chelsea Kidwell, Mackenzie Roman, and Danny Bae in cancer biology, mitochondrial biology, patient-derived cells, mitochondrial purification, 3D culturing techniques, and data analysis.
All 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: Cold Spring

Harbor Lab Tumor microenvironment and Metastasis Conference (October 2021 and August 2022), Myeloid Cells and Innate Immunity in Solid Tumors Keystone Meeting (September 2021), and the American Society for Cell Biology (December 2021).

How were the results disseminated to communities of interest?
Members of the lab have presented findings related to this work at the Cold Spring Harbor Lab Tumor microenvironment and Metastasis Conference (October 2021 and August 2022), and the American Society for Cell Biology (virtual, December 2021).
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. Manipulate reactive oxygen species generation in cancer cells: We had previously determined that transferred mitochondria accumulate reactive oxygen species (ROS) and we efficiently induced ROS with mito-killerred expressing cancer cells; however, our goal for the next reporting period is to efficiently quench ROS to test whether ROS is necessary and sufficient for changes in breast cancer cell behavior.
    a. Test a series of ROS quenchers 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
2. Determine whether purified macrophage mitochondria induce cancer cell proliferation through ROS accumulation: We have shown that bath application of purified macrophage mitochondria induces cancer cells proliferation. In the next reporting period, we will determine whether cancer cells that take up purified macrophage mitochondria exhibit ROS accumulation at exogenous mitochondria.
    a. Evaluate ROS accumulation with the Grx-roGFP2 biosensor
    b. Quench ROS with the addition of ROS scavengers, and quantify ROS with the Grx-roGFP2 biosensor

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
Nothing to report.

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.
Kidwell CU*, Casalini JR*, Pradeep S, Scherer S, Greiner D, Johnson JS, Olsen G, Rutter J, Welm A, Zangle T, **Roh-Johnson M**. (2021). Lateral macrophage mitochondrial transfer induces cancer cell proliferation through ROS signaling. *equal contribution. BioRxiv. under consideration at *Nature Cell Biology*.

Greiner D, Scott T, Olson GS, Aderem A, **Roh-Johnson M**, and Johnson J.S. (2022) Genetic modification of primary human myeloid cells to study cell migration, activation, and organelle dynamics. Current Protocols, *accepted*.

Nuebel E, Morgan JT, Fogarty S, Winter JM, Lettlova S, Berg JA, Chen YC, Kidwell CU, Maschek JA, Clowers KJ, Argyriou C, Chen L, Wittig I, Cox JE, **Roh-Johnson M**, Braverman N, Bonkowsky J, Gygi SP, Rutter J. (2021). The biochemical basis of mitochondrial dysfunction in Zellweger Spectrum Disorder. *EMBO Reports*, Aug 5:e51991.

Books or other non-periodical, one-time publications.
Nothing to report.

Other publications, conference papers and presentations.

<u>Oral Presentations at Conferences and Other Institutions:</u>

Kidwell CU, Casalini J, Johnson JS, **Roh-Johnson M**. Macrophage mitochondrial transfer to tumor cells. 2021. Myeloid cells Keystone Meeting, virtual.

Greiner D, Varady S, and **Roh-Johnson M**. Macrophage-dependent iron regulation in breast cancer. 2021. Myeloid cells Keystone Meeting, virtual

Casalini J, Kidwell CU, Johnson JS, **Roh-Johnson M**. Lateral macrophage mitochondrial transfer acts as a signaling source regulating proliferation in cancer cells. 2021. ASCB, 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 had made a number of constructs to visualize mitochondria and mitochondrial function in cells, and deposited these constructs on Addgene. These plasmids will become available 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)

Name:	Chelsea Kidwell
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	https://orcid.org/0000-0003-4269-2503
Nearest person month worked:	2
Contribution to Project:	Dr. Kidwell has performed all experiments with patient-derived xenograft organoids in 3D.
Funding Support:	No other direct funding support.

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 mitochondria bath application 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:	6
Contribution to Project:	Mr. Fierro assisted with quantification of reactive oxygen species.
Funding Support:	No other direct funding support.

Name:	Mackenzie Roman
Project Role:	Research Technician
Researcher Identifier (e.g. ORCID ID):	https://orcid.org/0000-0003-4788-8636
Nearest person month worked:	4
Contribution to Project:	Ms. Roman assisted with isolating primary macrophages, and transducing macrophages for downstream experiments.
Funding Support:	No other direct funding support.

Name:	Danny Bae
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	https://orcid.org/0000-0003-4788-8636
Nearest person month worked:	11
Contribution to Project:	Dr. Bae assisted with experiments related to quenching reactive oxygen species in recipient cancer cells.
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?
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:
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: 08/01/21 – 07/31/23

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