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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, macrophages, mitochondrial transfer, reactive oxygen species, proliferation

3. **ACCOMPLISHMENTS:**
What were the major goals of the project?
Below I have indicated our progress/accomplishments for each goal of the project during the 3-year award period.

Specific Aim 1: Manipulating macrophage mitochondrial dynamics and transfer to breast cancer cells	**Timeline**	**Completion Date/Progress**
Major Task 1 – Efficiently manipulate macrophage mitochondria morphology to determine effects on mitochondrial transfer to MDA-MB-231 breast cancer cells	Months	
Establish effective shRNA knockdown strategies in primary macrophages for use in subsequent aims	1-3	Initiated experiments in Sept 2019. Analysis occurred in Mar-Sep 2020. DRP1 shRNA finalized in December 2020 and confirmed with qRT-PCR (**Fig 3**).
Establish quantitative pipeline with analysis software to assess mitochondrial morphology	3-5	Initiated and optimized Mitochondrial Network Analysis (MiNA) in March 2020 during COVID lockdown. Analysis finalized in March 2021 for publication (**Fig 2&3**).
Determine whether changes in macrophage mitochondria morphology in Miro shRNA affect mitochondrial transfer with flow cytometry	5-7	Initiated experiments in Sept 2019. Analysis occurred in Mar-Sep 2020, when shRNA confirmation was occurring (Major task 1.1). DRP1 shRNA macrophages exhibit decreased mitochondrial transfer to breast cancer cells first confirmed in May 2020. Data

		finalized in May 2021 for publication (**Fig 2&3**).
Milestone Achieved: Ability to inhibit mitochondrial transfer to MDA-MB-231 breast cancer cells	7	All goals achieved. All experiments were completed by Feb 2021, and final figures for publication were completed by May 2021.
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	Initiated experiments in Sep 2019. Analysis occurred during Mar-Sep 2020 (along with shRNA experiments outlined in Major Task 1). Feb 2021 - confirmation of macrophage polarization using single markers (**Fig 1**).
Determine effects of macrophage polarization on mitochondrial transfer with flow cytometry	9-12	Began analyzing mitochondrial morphology in polarized macrophages with MiNA software in March 2020. Completed analysis by Feb 2021, and final figures were generated in April 2021 for publication. Determined that M2-like polarized macrophages exhibit increased mitochondrial transfer to breast cancer cells (**Fig 2**)
Establish coculturing methods for macrophages with patient derived xenograft organoids in 2D & 3D	11-15	Initiated coculturing experiments with patient-derived xenograft organoids in Sept 2020 in 2D. Experiments in 2D caused patient-derived xenograft organoid cells to die, so focused on 3D culturing methods. (**Fig 4A,B**)
Assess mitochondrial transfer to patient-derived xenograft organoids in 2D & 3D with flow cytometry	15-18	Initiated coculturing experiments in Sep 2020, experiments in 2D and 3D performed from Sep 2020-Feb 2021. Finalized analysis and figures by August 2021 - established mitochondrial transfer to patient-derived xenograft organoid cells in 3D (but not 2D) (**Fig 4C-E**)

Milestone Achieved: Determination of macrophage polarization conditions for efficient mitochondrial transfer to patient-derived xenograft organoids	18	All goals achieved. All experiments completed by Feb 2021, and final figures for publication completed by August 2021.
Specific Aim 2: Determine how transferred macrophage mitochondria affect MDA-MB-231 breast cancer cell behavior.	**Timeline**	**Institution**
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	Months	
Generate and assess the level of reactive oxygen species with biosensors and dyes at transferred mitochondria in MDA-MB-231 cells	18-20	Initiated experiments in December 2019 with reactive oxygen species dyes and biosensors, and continued through Oct 2020. Analysis occurred during Oct 2020-Feb 2021. Final figures for publication generated by August 2021 – determined reactive oxygen species accumulation at transferred mitochondria using Grx1 biosensor and Orp1 biosensor. (**Fig 5**)
Generate and assess the level of reactive oxygen species in primary macrophages with biosensors and dyes before mitochondrial transfer	19-22	We were unable to achieve this specific goal as expressing any biosensor in the macrophages led to cell lethality. Instead, we treated macrophages with H202, thereby resulting in increased ROS production in macrophages and observed increased mitochondrial transfer to cancer cells (**Fig 8E,F**)
Manipulate reactive oxygen species generation and downstream signaling with chemical inhibitors in MDA-MB-231 cells	21-23	We were unable to quench reactive oxygen species in cancer cells in the second year of this award. However, in the final year of the award, we were successfully able to quench ROS production in cancer cells that had taken up purified macrophage mitochondria. Goal achieved in Dec 2022 (Also, outlined in Major Task 2.2). (**Fig 8E,F**)

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 (**Fig 6**)
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	Initiated mitochondrial purification experiments in May 2019. And analyzed quality of the purification from May 2019-Feb 2020. These mitochondrial purifications were for the direct injections into cancer cells, as outlined in the DOD proposal. We later performed additional mitochondrial purification experiments beginning in Dec 2021 for mitochondrial bath applications (not proposed in DOD application, but requested by manuscript reviewers), and finalized approaches in January 2022 (**Fig 7&8**)
Test whether purified mitochondrial injections induce reactive oxygen species in MDA-MB-231 cells with biosensors	29-32	Began purified mitochondrial injections into cancer cells in June 2019, and continued experiments until Mar 2020. During the COVID-19 lockdown, we were unable to continue these experiments because they required collaboration with our Biochemistry colleagues. Additionally, the experiment was finicky and laborious.

		In Dec 2021, we switched to purified mitochondrial bath applications to cancer cells. December 2022 – could not definitively determine that increased reactive oxygen species in cancer cells upon uptake of purified mitochondria from macrophages, but were able to show that quenching ROS in cancer cells that had taken up purified macrophage mitochondria exhibited decreased proliferation (**Fig 7&8**)
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 December 2022 – showed more robustly that cancer cells that had taken up purified macrophage mitochondria retained proliferative capacity (**Fig 7&8**)
Milestone(s) Achieved: Determining whether transferred mitochondria alone can drive metastatic potential of MDA-MB-231 cells	36	December 2022 – Achieved.

What was accomplished under these goals?
Below I describe our accomplishments for each year of the award, and how they relate to the goals as outlined in our statement of work.

Year 1 (08/01/2020 – 07/31/2021):
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.

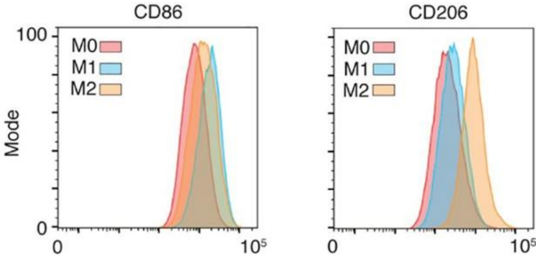

Fig. 1: Macrophage polarization. Macrophages were activated with IFN-γ (M1 activation; left) or IL-4/IL-13 (M2 activation; right) for 48 hours and flow cytometry was used to determine expression of canonical M1 (CD86, left) and M2 (CD206, right) markers. Representative histograms shown.

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.

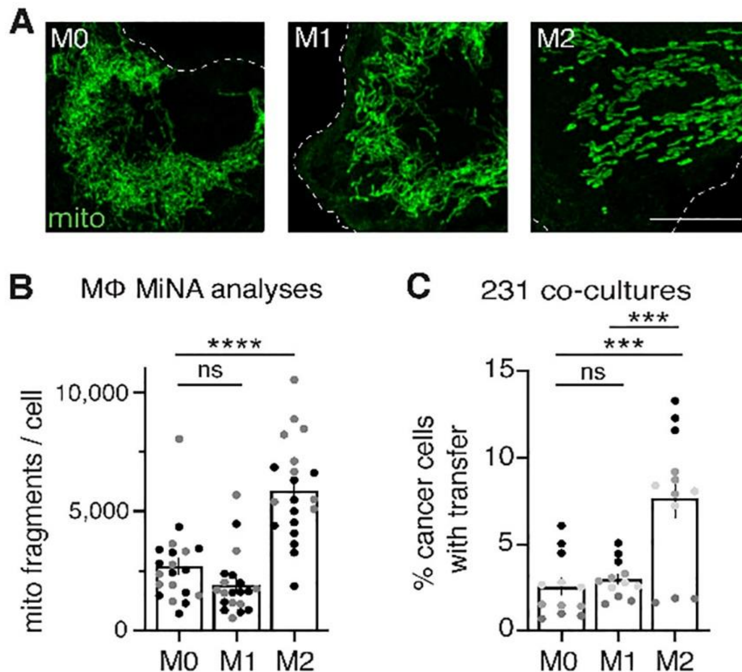

Fig. 2. M2-like macrophage activation increases mitochondrial fragmentation and transfer to breast cancer cells. (A) Representative images of mito-mEm+ macrophages that were non-stimulated (M0, left) or activated to become M1-like (middle) or M2-like (right) for 48 hours. (B) Mitochondrial network analyses (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).

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).

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

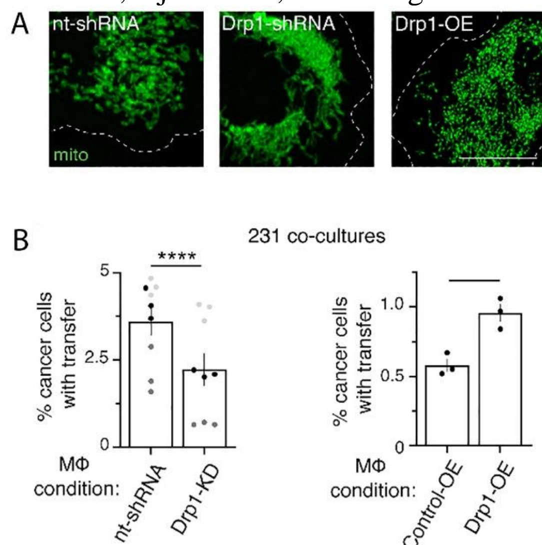

Fig. 3. Increased mitochondrial fragmentation increases transfer. (A)Representative images of mito-mEm (green) macrophages transduced with lentiviruses (upper left corner). (B) Rates of mitochondrial transfer with macrophages with Drp1-knockdown (KD; left) or Drp1-overexpression (OE; right) compared to appropriate controls (N=3). 1-way ANOVA

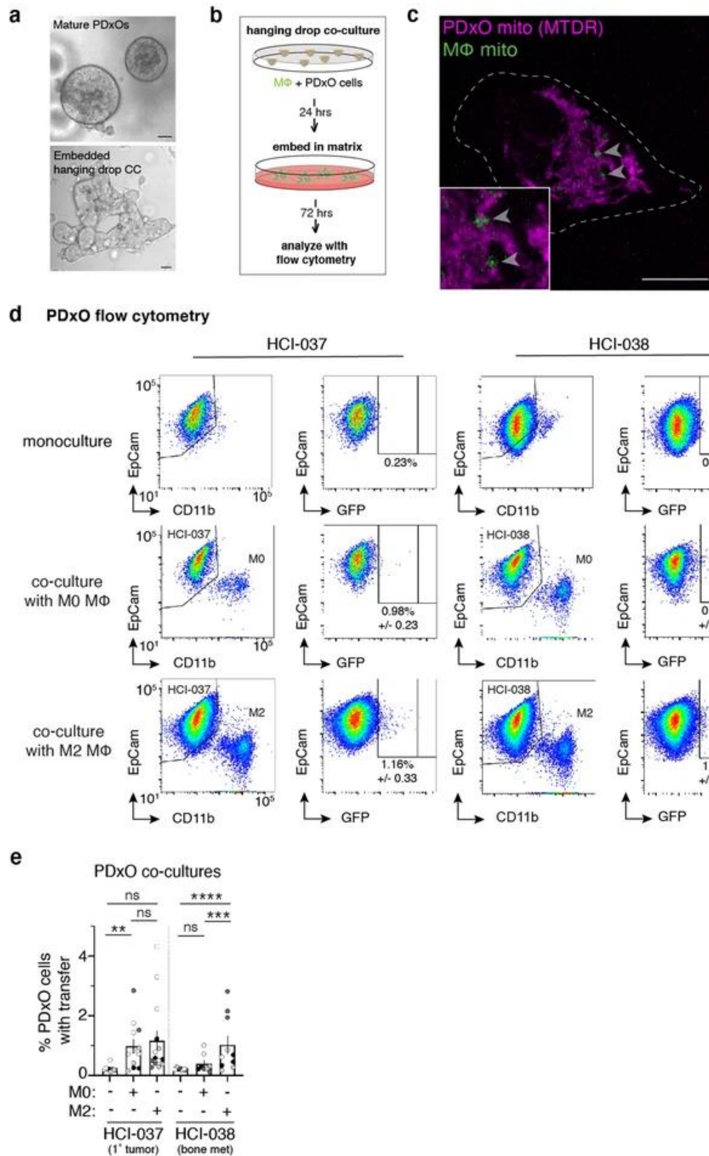

Figure 4: Macrophage transfer mitochondria to patient-derived cells. (a) Representative images of the HCI-037 patient-derived xenograft organoid (PDxO) line in culture (top) or an embedded hanging drop co-culture with macrophages (bottom). (b) Schematic of experimental setup of PDxOs (gray)/mito-mEm macrophages (green) co-cultures. Co-cultures are plated in suspended drops of media (hanging drops) to allow for the formation of cell aggregates without adherence to a substrate. After 24 hr, the co-cultured cells are embedded into a matrix (Matrigel) and cultured for 72 hr before analysis with flow cytometry. (c) Representative image of a FACS-isolated PDxO cell containing macrophage mitochondria (green, arrowhead) that are MTDR-negative. (d) Top row: Representative flow cytometry plot of PDxO monocultured cells used as the experimental control when quantifying mitochondrial transfer. Middle row: PDxO cells co-cultured with mito-mEm-expressing M0 macrophages. Bottom row: PDxO cells co-cultured with mito-mEM expressing M2-like macrophages. PDxO lines used for co-culture are indicated at the top of the corresponding panels. Scale bars are 10 µm. (e), Rate of mitochondrial transfer to HCI-037 (left 3 columns) or HCI-038 (right 3 columns) PDxO cells from M0 or M2 macrophages (each dot is one replicate, N=4 donors). Two-way ANOVA, **p<0.01; ***p<0.001; ****p<0.0001

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.

In the first progress report, I did not provide data with the PDXo, so I am providing it here in this final report. We first cocultured PDXos with macrophages in 3D, but the PDXo cells died, so we

moved to 3D approaches. We first grew the PDXo as organoids in 3D (**Fig. 4A**), dissociated these cells and cocultured them with macrophages as a hanging drop suspended on the lip of a tissue culture dish (**Fig. 4A,B**). These hanging drop cocultures were then embedded in matrigel for 72 hours, and macrophage mitochondrial transfer was analyzed by flow cytometry. We first determined that macrophages transfer mitochondria to PDxO cells by microscopy (**Fig 4C**). We then determined that M2-like macrophages transferred mitochondria to PDxO cells more efficiently than M1-like or M1 macrophages (**Fig 4D,E**).

We met all of the goals proposed in the research plan in the first reporting year.

Year 2 (08/01/2021 – 07/31/2022):

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.

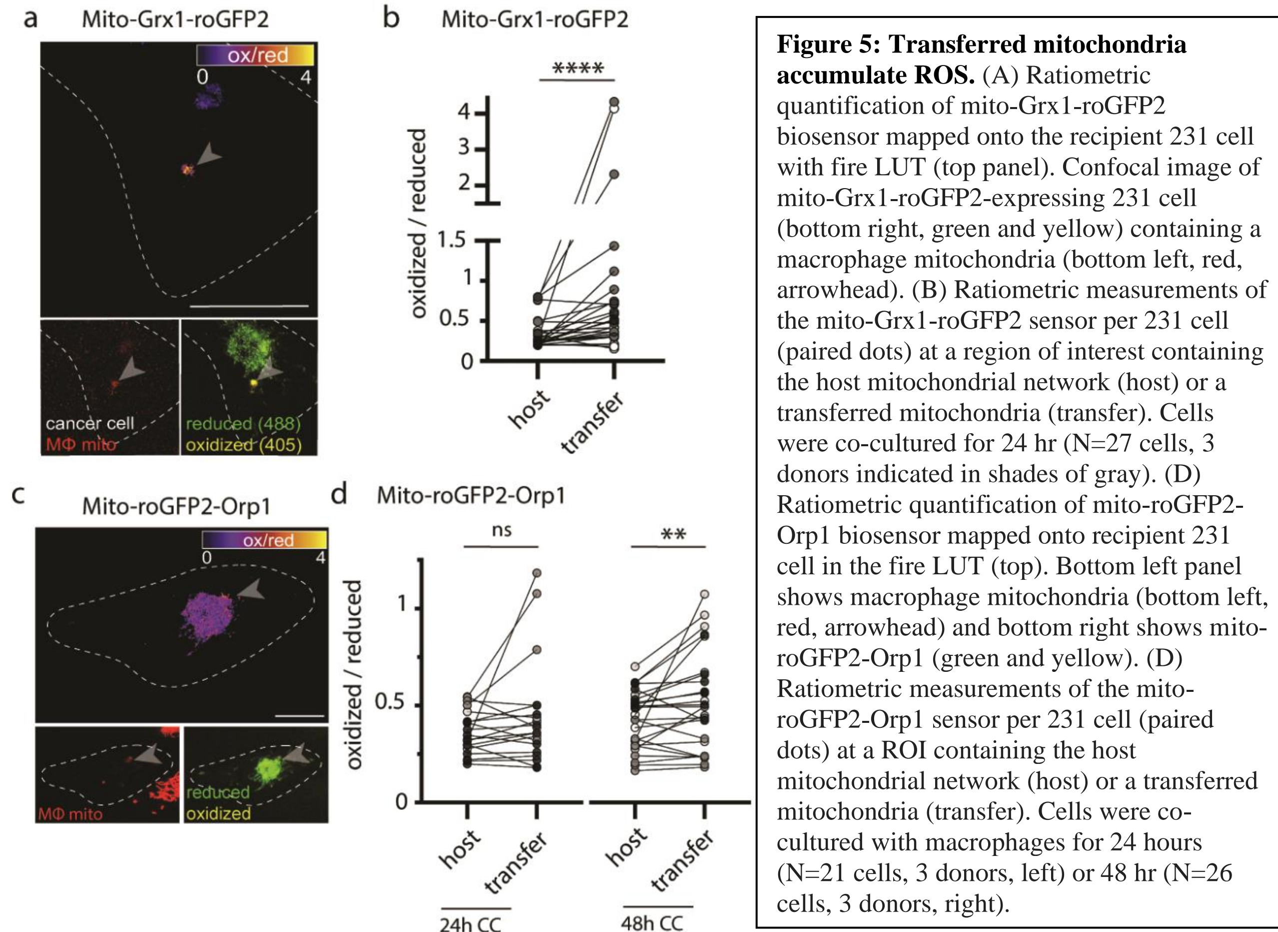

Figure 5: Transferred mitochondria accumulate ROS. (A) Ratiometric quantification of mito-Grx1-roGFP2 biosensor mapped onto the recipient 231 cell with fire LUT (top panel). Confocal image of mito-Grx1-roGFP2-expressing 231 cell (bottom right, green and yellow) containing a macrophage mitochondria (bottom left, red, arrowhead). (B) Ratiometric measurements of the mito-Grx1-roGFP2 sensor per 231 cell (paired dots) at a region of interest containing the host mitochondrial network (host) or a transferred mitochondria (transfer). Cells were co-cultured for 24 hr (N=27 cells, 3 donors indicated in shades of gray). (D) Ratiometric quantification of mito-roGFP2-Orp1 biosensor mapped onto recipient 231 cell in the fire LUT (top). Bottom left panel shows macrophage mitochondria (bottom left, red, arrowhead) and bottom right shows mito-roGFP2-Orp1 (green and yellow). (D) Ratiometric measurements of the mito-roGFP2-Orp1 sensor per 231 cell (paired dots) at a ROI containing the host mitochondrial network (host) or a transferred mitochondria (transfer). Cells were co-cultured with macrophages for 24 hours (N=21 cells, 3 donors, left) or 48 hr (N=26 cells, 3 donors, right).

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. 5A, 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. 5C, D**) after 48 hours of co-culture. At 24 hours, we observed a similar trend, but no statistically significant difference (**Fig. 5D**). 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. 6A**). 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. 6B,C**). These results indicate that ROS induction alone is sufficient to promote cancer cell proliferation.

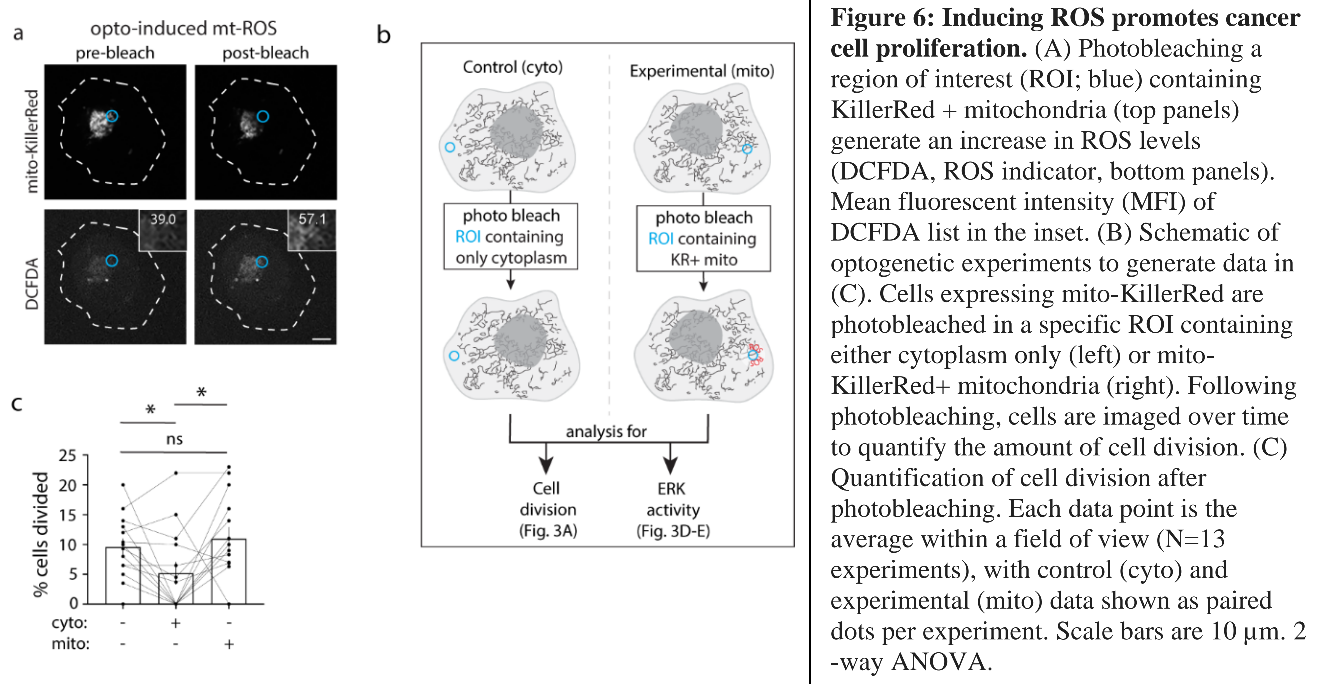

Figure 6: Inducing ROS promotes cancer cell proliferation. (A) Photobleaching a region of interest (ROI; blue) containing KillerRed + mitochondria (top panels) generate an increase in ROS levels (DCFDA, ROS indicator, bottom panels). Mean fluorescent intensity (MFI) of DCFDA list in the inset. (B) Schematic of optogenetic experiments to generate data in (C). Cells expressing mito-KillerRed are photobleached in a specific ROI containing either cytoplasm only (left) or mito-KillerRed+ mitochondria (right). Following photobleaching, cells are imaged over time to quantify the amount of cell division. (C) Quantification of cell division after photobleaching. Each data point is the average within a field of view (N=13 experiments), with control (cyto) and experimental (mito) data shown as paired dots per experiment. Scale bars are 10 µm. 2 -way ANOVA.

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. 7**). 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. 7A**), and do not exhibit membrane potential (**Fig. 7B**). 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. 7C**, comparing black bars in lanes 1&2). These results indicate that transferred mitochondria promote proliferation in cancer cells.

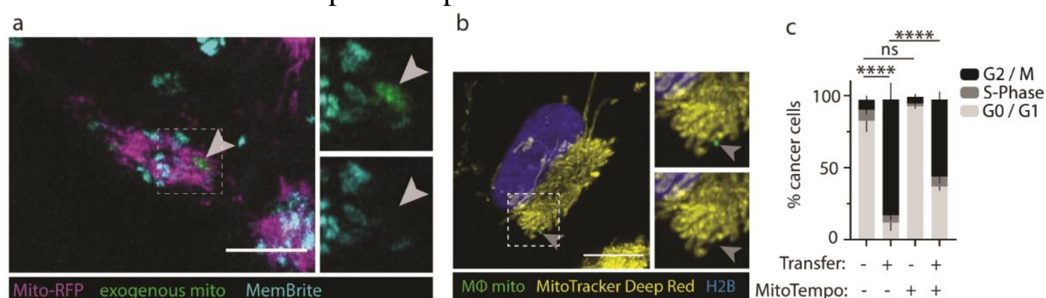

Figure 7: Exogenous macrophage mitochondria are not encapsulated by a membrane, are depolarized, and promote cancer cell proliferation. (A) Exogenous macrophage mitochondria (green) are negative for a membrane dye, MemBrite (cyan), arrowheads. (B) Exogenous macrophage mitochondria (green) are MitoTracker Deep Red negative (yellow), arrowhead). Cancer cells that take up purified macrophage mitochondria exhibit an increase in proportion of cells in the G2/M phase of the cell cycle (black bars), that is ameliorated in the presence of MitoTEMPO, a ROS scavenger. 2-way ANOVA, ****$p<0.001$.

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 (**Fig. 7C**), but we are still struggling to quench ROS during the in vitro macrophage mitochondrial transfer assays. We completed the rest of the goals outlined in year 2 of the award.

Year 3 (08/01/2022 – 07/31/2023)

In the final year of this award, we were able to spend the remaining funds within 5 months:
Major Activities – The main activity during this reporting period was to complete the project and have the paper describing this project accepted for publication.

Specific Objectives – We sought to complete an experiment within major task 2 of specific aim 2, which was to definitively determine whether macrophage *mitochondria* drove the proliferation in cancer cells versus other components that may be transferred along with macrophage mitochondria. This was a key aspect of the project required for publication. We also sought to finalize the manuscript for publication.

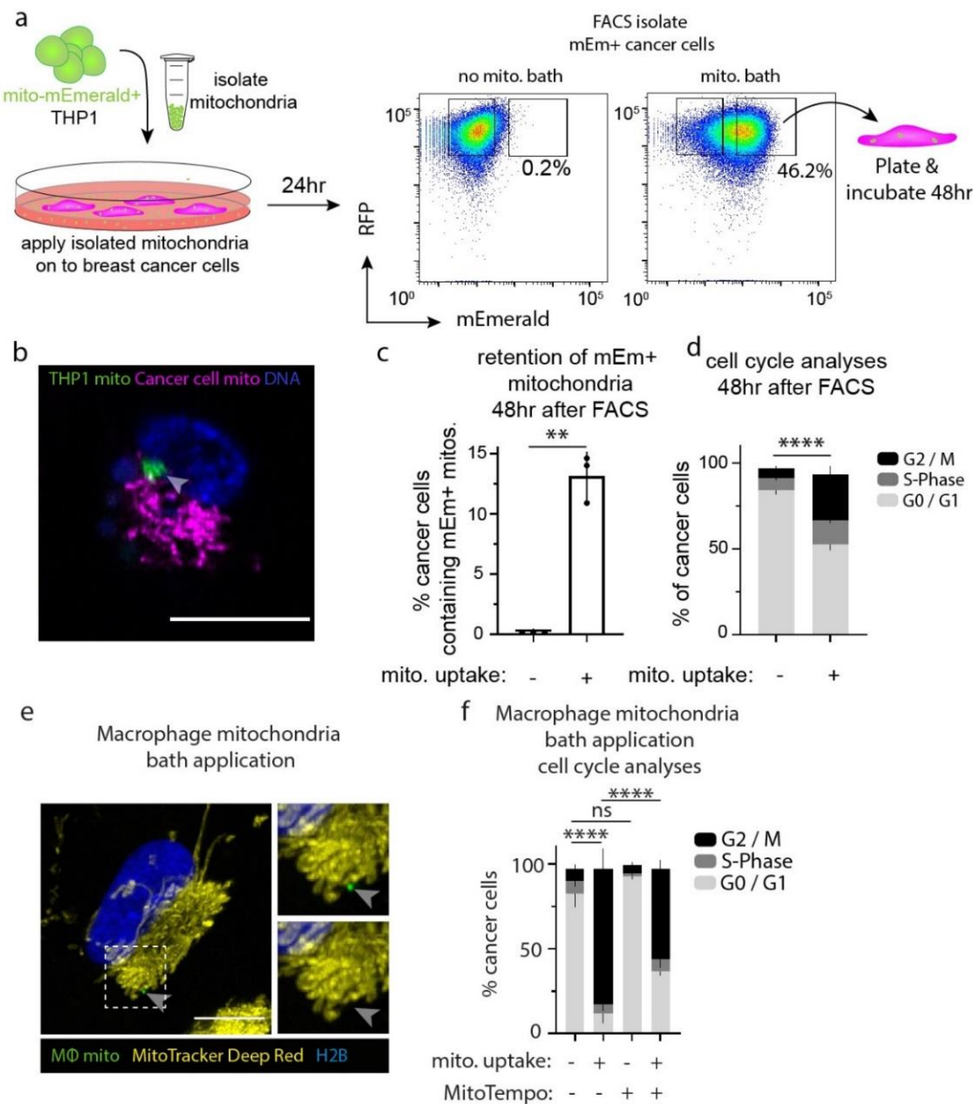

Figure 8 (A) Schematic of mitochondrial solation and bath application on MDA-MB-231 cells. Mitochondria are isolated from mito-mEmerald expressing THP-1 monocytes and bath applied at 20–30 µg/mL for 24 hr. Cancer cells which had taken up mEm+ mitochondria are then FACS-isolated and plated for 48 hr for further analyses. (B) Representative confocal image showing mito-RFP-expressing 231 cell (magenta) that had taken up macrophage mitochondria (green, grey arrow). (C) 48 hr after FACS-isolating 231 cells with macrophage mitochondria, flow cytometry was used to determine percent of daughter cells which still contain mEm+ mitochondria. N=3 biological replicates. (D), Cell cycle analysis of daughter cells 48 hr after FACS-isolation of 231 cells that had taken up macrophage mitochondria. N=3 biological replicates. (E) Exogenous purified macrophage mitochondria (green) are void of mitochondrial membrane potential (MitoTracker Deep Red-negative, yellow, arrowhead) in cancer cells. (F) Cell cycle analysis of cancer cells with exogenous purified macrophage mitochondria versus sister cells that did not take up exogenous purified mitochondria, either treated with vehicle or 100 µM mitoTEMPO (mitochondrially-targeted superoxide scavenger. N=3 donors; statistics for G2/M only). For all panels, SEM is displayed and scale bars are 10 µm. Welch's t-test (C), 2-way ANOVA (D,F). $^{**}p<0.01$, $^{****}p<0.0001$.

Significant Results/Key Outcomes – We performed experiments to rigorously test whether transferred macrophage mitochondria cause cancer cell proliferation, rather than mitochondrial receipt and proliferation being correlative events in cancer cells. We also wanted to determine whether the observed proliferative phenotype is due to macrophage mitochondria, and not other molecules that are passed along with the macrophage mitochondria. Thus, we biochemically purified mitochondria from a macrophage cell line, THP-1, and directly applied these macrophage mitochondria to cancer cells for 24 hr (**Fig 8A**). We then FACS-isolated cancer cell populations that contained purified macrophage mitochondria, and allowed this population to undergo additional rounds of cell division, and then reanalyzed the proliferative capacity of cancer cells that had

retained the macrophage mitochondria versus cancer cells that had lost the macrophage mitochondria over this time. We first confirmed that cancer cells retained the macrophage mitochondria by imaging (**Fig 8B,C**). We also found that cancer cells that had retained the macrophage mitochondria exhibited an increased percentage of cells in the G2/M phase of the cell cycle compared to cancer cells that had lost the macrophage mitochondria (**Fig 8D**). These results suggest that the proliferative capacity is retained in cancer cells that receive macrophage mitochondria. We also found that the purified macrophage mitochondria in cancer cells lacked membrane potential (**Fig 8E**), and that the proliferative increase observed in these cells was ameliorated when ROS was quenched with a mitochondrially localized superoxide scavenger, mitoTEMPO (**Fig 8F**; comparing black bars in lanes 2&4). Importantly, cancer cells that did not internalize mitochondria were not affected by ROS quenching (**Fig 8F**; comparing black bars in lanes 1&3). These results indicate that transferred mitochondria promote proliferation in a ROS-dependent manner.

4) Other Achievements: The final manuscript describing this work was submitted in December 2022, and accepted for publication in March 2023.

➔ Kidwell, CU*, Casalini JR*, Pradeep S, Scherer S, Greiner D, Bayik D, Watson DC, Lathis JD, Olsen G, Johnson JS, Rutter J, Welm A, Zangle T, **Roh-Johnson M#**. Transferred mitochondria accumulate reactive oxygen species, promoting proliferation. (2023). *eLife*, Mar 6; 12:e85494. PMCID:PMC10042539.

What opportunities for training and professional development has the project provided?
Year 1: This project 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 also attended 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).

Year 2: 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.

Year 3: During the final reporting period, this project provided extensive research training to Joey Casalini (PhD student, funded by F31 NRSA), Daniel Greiner (PhD student), Julio Fierro (PhD student), and Mackenzie Roman (research technician) in cancer biology, primary macrophage isolation, mitochondrial purification, flow cytometry, and data analysis. Joey Casalini and Daniel Greiner were also involved in the writing of the manuscript that was accepted for publication. Daniel Greiner and Julio Fierro attended the American Society for Cell Biology conference in Dec 2022 to disseminate knowledge and train in scientific communication. Joey Casalini attended and

presented this work at the Cold Spring Harbor Lab Tumor Microenvironment and Metastasis Conference in August 2022.

How were the results disseminated to communities of interest?
Year 1: 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.

Year 2: 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.

Year 3: Members of the lab presented findings related to this work at the American Society for Cell Biology in December 2022 and the Tumor Microenvironment and Metastasis Conference in August 2022. Minna Roh-Johnson participated in K-12 outreach, including visiting local elementary schools and participating in Skype a Scientist in 2022.

What do you plan to do during the next reporting period to accomplish the goals?
Nothing to Report

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.

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.

5. PRODUCTS:

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

Journal publications.
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. PMID: 34351705 (acknowledgement of federal support – yes).

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. Curr Protoc. 2022 Aug;2(8):e514. doi: 10.1002/cpz1.514. PMID: 36018279. (acknowledgement of federal support – yes).

Kidwell, CU*, Casalini JR*, Pradeep S, Scherer S, Greiner D, Bayik D, Watson DC, Lathia JD, Olsen G, Johnson JS, Rutter J, Welm A, Zangle T, **Roh-Johnson M#**. Transferred mitochondria accumulate reactive oxygen species, promoting proliferation. (2023). *eLife*, Mar 6; 12:e85494. PMID: 36876914 (acknowledgement of federal support – yes).

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. 2020. 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. 2020. Horizontal transfer of macrophage mitochondria in cancer. *Reed College*, virtual.

Kidwell CU, Casalini J, Johnson JS, Roh-Johnson M. 2020. Horizontal transfer of macrophage mitochondria during metastasis. *University of Michigan,* Student-invited speaker, virtual

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. *American Society for Cell Biology*, virtual.

Casalini J, Kidwell C, Johnson JS, Roh-Johnson M. Lateral transfer of macrophage mitochondria promotes ERK-dependent cancer cell proliferation. 2022. *Cold Spring Harbor Lab Tumor Microenvironment and Metastasis Conference*. Oral Presentation. Cold Spring Harbor, NY.

Greiner D, Varady S, and Roh-Johnson M. Macrophage-dependent iron regulation in breast cancer. 2022. Poster presentation, *American Society for Cell Biology*, Washington DC.

- **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 should now be available since the manuscript describing these constructs is published.

6. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Year 1:

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.

Year 2:

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.

Year 3:

Name: Minna Roh-Johnson
Project Role: PI
Researcher Identifier (e.g. ORCID ID): https://orcid.org/0000-0003-3961-4547
Nearest person month worked: 1

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: Joey Casalini
Project Role: Graduate Student
Researcher Identifier (e.g. ORCID ID): https://orcid.org/0000-0002-3515-3248
Nearest person month worked: 5
Contribution to Project: Mr. Casalini assisted with experiments related to purified macrophage mitochondria, quenching reactive oxygen species in recipient cancer cells.
Funding Support: F31 NRSA

Name: Daniel Greiner
Project Role: Graduate Student
Researcher Identifier (e.g. ORCID ID): https://orcid.org/0000-0001-6272-3237
Nearest person month worked: 5
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: 5
Contribution to Project: Mr. Fierro supported macrophage isolation procedures.
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: 5
Contribution to Project: Ms. Roman assisted with isolating primary macrophages, and transducing macrophages for downstream experiments.
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.

7. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: N/A

QUAD CHARTS: N/A

8. APPENDICES:

Award chart attached.