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TITLE: Identification and Targeting of Metastasis-Suppressing miRNAs in Triple-Negative Breast Cancer

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14. ABSTRACT

Background. Triple-negative breast cancer (TNBC) constitutes ~20-25% of all breast cancer cases and has the worst prognosis due to high rates of distant recurrence, high rates of metastasis and a lack of effective molecularly targeted therapies. For patients diagnosed with advanced TNBC, the median duration of survival is only 12 months, which is much shorter than for patients diagnosed with other types of breast cancer. Therefore, TNBC patients, especially those presenting with advanced stage disease, are in desperate need of novel molecularly targeted therapies. Since TNBC is heterogeneous, the molecular underpinnings have been difficult to discern, thus making it challenging to develop effective targeted therapeutics. miRNA replacement therapy is a novel type of targeted therapy that is beginning to be investigated in clinical trials as it shows great promise. The remaining hurdle, however, is uncovering the most effective method for *in vivo* delivery. One mechanism for delivery that has not been previously investigated is a liposome-like structure called a DNAsome, which is ideal for the *in vivo* delivery of small RNA molecules. We therefore hope to develop miRNA replacement therapy for the regression of existing metastases in late-stage TNBC patients using the DNAsome.
Overarching Challenge. This proposal aims to 1) revolutionize treatment regimens by replacing interventions that have life-threatening toxicities with molecularly targeted ones that are safe and effective and 2) Eliminate the mortality associated with metastatic breast cancer.
Objective/Hypothesis. miRNA dysregulation has been implicated in tumorigenesis and metastasis within various cancer types, including breast cancer. Importantly, we have shown as proof-of-principle, that *in vivo*, miR-708, an anti-metastatic miRNA, can block metastatic progression following metastatic seeding of TNBC cells. *We therefore hypothesize that there are miRNAs that initiate metastasis regression and/or block metastatic progression. We also propose that these miRNA can be delivered in vivo as part of an effective cancer treatment.* Our overall goal is use preclinical models of breast cancer to demonstrate whether miRNA replacement therapy is an effective way of initiating the regression of existing breast cancer metastases and thus reducing the high mortality rate characteristic of TNBC.
Specific Aims. 1) To identify miRNAs that are capable of initiating regression of metastases in TNBC and 2) To assess the potential of DNAsome delivered miRNAs as a metastasis regression therapeutic.
Study Design. We have identified many miRNAs that are downregulated in metastatic TNBC cell lines compared to non-metastatic but tumorigenenic breast cancer cell lines. We propose to perform an *in vivo* screen to identify if any of these miRNAs can specifically regress existing lung, brain or bone metastases. We will then identify the mRNA targets of each of the miRNAs that leads to regression of metastases at any or all of these sites. This will help elucidate the mechanism by which the miRNA of interest leads to the regressin of existin metastases. Together with the laboratory of Dr. Dan Luo at Cornell University, we will create DNAsomes containing one or more of the miRNAs of interest and optimize *in vivo* delivery of the DNAsomes. Finally, we will use a patient-derived xenograft mouse model of TNBC to test the efficacy of DNAsome mediated miRNA replacement therapy in terms of lung, brain and bone metastasis regression. Altogether, these experiments aim to identify the molecular mechanisms behind TNBC metastasis and develop miRNAs replacement therapy is an effective treatment for existing metastases in TNBC mouse models.
Impact. This study has the potential to provide important preclinical data for the development of miRNAs as novel therapies for TNBC, a subtype of highly metastatic breast cancer with the worst prognosis and no viable treatment options. The implications

15. SUBJECT TERMS
Triple negative breast cancer, metastasis, micro RNA, miRNA, epigenetic, DNAsomes, nanoparticle, Targeted therapy

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Table of Contents

Page

1. **INTRODUCTION:**

This proposal aims to identify miRNAs that regress established metastasis and can be delivered for the treatment of advance high-risk breast cancer patients. We will pursue our goals by identifying miRNAs that lead to the regression of existing metastases, determining the molecular mechanisms by which the identified miRNAs induce regression and exploring the potential for in vivo delivery of miRNAs for the treatment of late-stage metastatic breast cancer. The projects two specific aims are (1) To identify miRNAs that regress established metastases in breast cancer and (2) To assess the potential of DNAsome delivered miRNAs as a metastasis regression therapeutic.

The overarching goals are to 1) Revolutionize treatment regimens by replacing interventions that have life-threatening toxicities with ones that are molecularly targeted, safe and effective and 2) Lead to the elimination of mortality associated with metastasis in high-risk category of breast cancer patients.

2. **KEYWORDS:**
Triple negative breast cancer, metastasis, micro RNA, miRNA, epigenetic, DNAsomes, nanoparticle, Targeted therapy

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

Aim1: To identify miRNAs that regress established metastases in breast cancer.

Major Task 1: Identification miRNAs that regress existing metastases in TNBC

Subtask 1: Create stable breast cancer cell lines expressing each miRNA of interest under the control of an inducible promoter. Cell lines used: MDA-MB-231-LM2, MCF7

Subtask 2: Determine whether expression of the miRNA of interest in cancer cells can regress existing lung metastases in an orthotopic mouse model of breast cancer. Total Animals = 100; SCID mice: n = 10 / miRNA expressing cell line x 10 miRNAs = 100 mice

Subtask 3: Generate breast cancer cell lines that specifically metastasize to the brain or bone that stably express each miRNA of interest under the control of an inducible promoter. Cell lines used: BoM-1833, BrM-831

Subtask 4: Determine whether expression of the miRNA of interest in cancer cells can regress existing brain and/or bone metastases in mice. Total Animals = 100

BALB/c-nu/nu nude mice: n=10 mice/miRNA expressing cell line (5 induced, 5 uninduced) x 10 miRNAs = 100 mice

Milestones: 1) Stable cells with inducible miRNAs, 2) Identification of 3-4 miRNAs that regress lung, brain and/or bone metastases in vivo. Local IRB/IACUC Approval; HRPO/ACURO Approval;

Major Task 2: mRNA target identification of miRNAs that show regression of lung, brain and/or bone metastases.

Subtask 1: Use of established algorithms to identify candidate mRNA targets of miRNAs identified in major task 1.

Subtask 2: Cloning of 3' UTRs of candidate mRNAs into a dual luciferase UTR vector.

Subtask 3: Generation of breast cancer cell line stably knocked down for the putative mRNA target(s). Cell lines used: MDA-MD-231-LM2

Subtask 4: *in vivo* analysis of whether the loss of mRNA target expression regresses established metastases. Total Animals = 100; SCID mice: n=10/miRNA or shRNA x 10 miRNAs (including scrambled controls) = 100 mice

Milestones: 1) Identification of several potential mRNA targets of the miRNA(s) of interest, 2) successful cloning of 3' UTR dual luciferase plasmids and subsequent identification of putative mRNA targets, 3) Generation of TNBC cell lines stably expressing an inducible shRNA construct for the putative mRNA target, 4) Verification that knock-down of the putative mRNA in cancer cells leads to the regression of established metastases.

Major Task 3: To evaluate the clinical significance of identified miRNAs.

Subtask 1: Use human TNBC patient samples to determine whether the miRNAs of interest are downregulated in metastatic lesions compared to the primary tumor.

Subtask 2: Use the same human TNBC patient samples as above to determine whether the mRNA targets are upregulated in metastatic lesions compared to the primary tumor.

Milestones: 1) Demonstration that the miRNAs of interest are downregulated in metastatic lesions compared to the primary tumor. 2) Demonstration that the mRNA target is upregulated in metastatic lesions compared to the primary tumor.

Aim 2: To assess the potential of DNAsome delivered miRNA as metastasis suppressing and regressing therapeutics.

Major Task 1: Synthesize DNAsomes carrying each miRNA shown to regress metastases in aim 1.

Milestone: As explained in the last report, gold nanoparticles will be utilized as carriers for delivery of miRNA.

Major Task 2: Determine whether DNAsomes carrying the miRNAs identified in aim 1 can regress in vivo metastases to the lung, brain and/or bone.

Subtask 1: Optimize the in vivo delivery of DNAsomes using PDX models of TNBC. Total Animals = 120 mice; SCID mice: n=5/group (4 doses, 3 delivery frequencies) = 60 mice/miRNA x 2 miRNAs (1 specific + 1 scrambled control) = 120 mice.

Subtask 2: Determine whether DNAsome mediated delivery of miRNAs can regress existing lung, brain and/or bone metastases. Total Animals = 100 mice; SCID mice: n=10/miRNA analyzed x 10 miRNAs = 100 mice

Subtask 3: Evaluation of toxicity in DNA-some-mediated delivery of miRNAs. Total Animals = 30 mice; Mice: n=15/group x 2 groups (DNAsome treated and untreated) = 30

Milestones: Demonstrate that miRNA replacement therapy can regress TNBC metastases in mouse models and *in vitro* toxicity will be evaluated.

What was accomplished under these goals?

For this reporting period, we are reporting progress for the following:

1) Aim 1, Major Task 1, Subtask 1
2) Aim 1, Major Task 1, Subtask 2
3) Aim 2, Major Task 2, Subtask 2

Aim 1. Major Task 1, Sub Task 1

In the last progress report, we had indicated that cloning miRs into doxycycline (dox)-inducible lentiviral vector expressing transactivator rtTA2 had issues with the leakiness of the inducible vector, as GFP+ cells were detected in the absence of the inducer dox We have resolved this by cloning the miR sequences (indicated in the first report) including Scrambled (Scr), miR-141-3p, miR-429 (Fig. 1A) in the third generation reverse tetracyclin-controlled transactivator 3 (rtTA3) expression vector (Fig. 1B). As shown, LM2 cells stably expressing the miRs showed a tight inducible expression (Fig 1C).

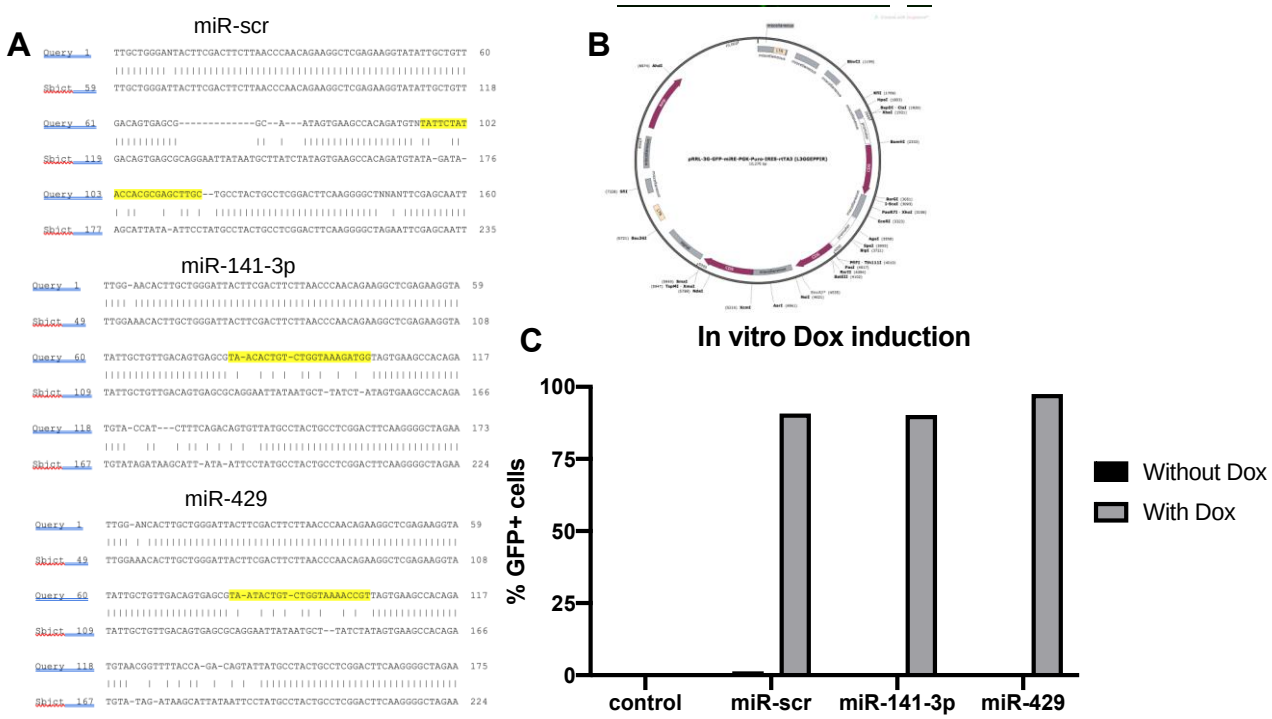

Figure 1. miR cloning. (A) Sequence of the cloned miR fragments (B) Map of the third generation reverse tetracycline controlled transactivator 3 (rtTA3) lentiviral expression vector. (C) GFP expression is induced after 24h of treatment with doxycycline

Plans for NCE: LM2 cells stably expressing dox-inducible miRNAs will be used for studies described in Aims 1 and 2.

Aim 1, Major task 1, Subtask 2.

To determine if expression of the selected miRNAs in cancer cells can regress lung metastases, we injected LM2 cells with stably integrated miRNAs (see Fig. 1) in tail veins in

SCID mice. After lung mets were established (week 2 determined by BLI), doxycycline feed was provided and lung mets were monitored by BLI (Fig. 2A). Compared to Scr controls, we observed a reduction in mets in LM2 cells expressiing either miR141-3p and miR-429 (Fig. 2B). Consistent with reduction in tumor reduction flow cytometry showed a reduction in percent mcherry+ tumor cells with miR-429 (Fig. 2C).

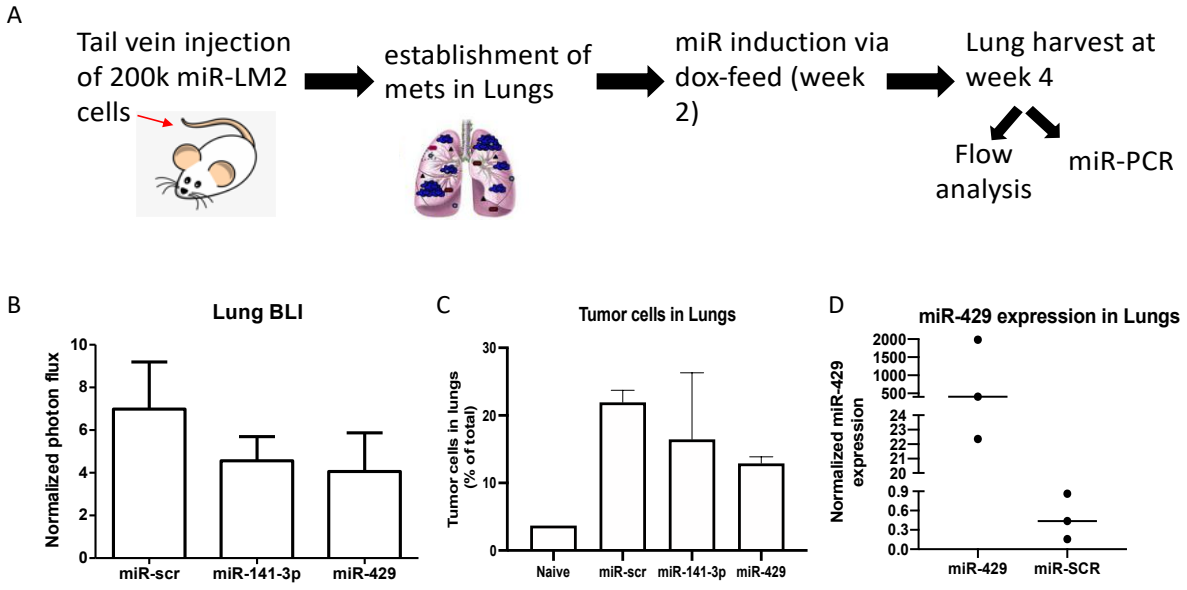

Figure 2. *In vivo* metastasis model. (A) *In vivo* model scheme. (B) Lung BLI at week 4 before harvest. (C) Percentage of mCherry+ tumor cells in lungs per total cells analyzed by flow. (D) miR-429 expression in lungs as determined by miR-PCR.

Plans for NCE: Determine whether miRNA expression in cancer cells can regress lung metastases in an orthotopic mouse model of breast cancer.

Aim 2, Major Task 2, Subtask 2: Determine whether DNAsome mediated delivery of miRNAs can regress existing lung, brain and/or bone metastases.

In the last report, we had showed that multilayered nanoparticles (NPs) carrying the metastasis suppressor microRNA miR-708 (miR708-NP) localize to orthotopic primary TNBC, and efficiently deliver the miR-708 cargo to reduce lung metastasis. Using a SOX2/OCT4 promoter reporter, we identified a population of miR-708^{low} cancer cells with tumor-initiating properties, enhanced metastatic potential and marked sensitivity to miR-708 treatment. *In vivo*, miR708-NP directly targeted the SOX2/OCT4-mCherry+ miR-708^{low} tumor cells to impair metastasis.

These studies are now published:

Ramchandani D, Lee SK, Yomtoubian S, Han MS, Tung CH, Mittal V. (2019) Nanoparticle Delivery of miR-708 Mimetic Impairs Breast Cancer Metastasis. Mol Cancer Ther. 2019 Mar;18(3):579-591.

Plans for NCE: We will use the optimized nanoparticle approach to test the consequences of miRNA expression in impacting established metastasis.

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

Opportunities for training and professional development on the project include the mentorship of post-doctoral associates to help advance their careers.

How were the results disseminated to communities of interest?
A paper was accepted for publication in early 2019:

Ramchandani D, Lee SK, Yomtoubian S, Han MS, Tung CH, Mittal V. (2019) Nanoparticle Delivery of miR-708 Mimetic Impairs Breast Cancer Metastasis. Mol Cancer Ther. 2019 Mar;18(3):579-591.

Also, in both 2018 and 2019, Dr. Mittal and Dr. Ramchandani have given invited seminars at the PSOC of Cornell University (Ithaca, NY) and the Keystone Symposium (Galveston, TX).

What do you plan to do during the next reporting period to accomplish the goals?
During the NCE period, we hope to investigate the following:

1) LM2 cells stably expressing dox-inducible miRNAs will be used for studies described in Aims 1 and 2.
2) Determine whether miRNA expression in cancer cells can regress lung metastases in an orthotopic mouse model of breast cancer.
3) We will use the optimized nanoparticle approach to test the consequences of miRNA expression in impacting established metastasis.

Our main goal is to identify miRNAs as novel therapeutic agents for reducing established mets.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?
Our study has the potential to establish the potential of new miRNAs as an attractive approach for treatment of metastatic breast cancer including TNBC using a novel delivery method.

What was the impact on other disciplines?
Progress in identifying novel miRNAs as therapeutic agents for metastatic TNBC is likely to attract many investigators across disciplines in breast cancer research and result in rapid advancements towards finding a potential therapy.

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:

Nothing to Report

6. PRODUCTS:

Nothing to Report

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

What individuals have worked on the project?

Name:	*Vivek Mittal (PD/PI - WCM) – 11% Effort*
Project Role:	*PD/PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1.32*
Contribution to Project:	*Dr. Mittal oversees all aspects of the proposal as PD/PI. FYI: Dr. Mittal was a Mentor to Dr. Havel on the project from 04/15/16 – 07/15/16 and assumed the role of PD/PI from 07/16/16 – onwards, after Dr. Havel left on 07/15/16 to pursue other opportunities at a non-academic institute. Dr. Mittal will remain the PD/PI for the remainder of the proposal.*
Funding Support:	

Name:	*Divya Ramchandani, PhD (Post-Doc - WCM) – 50% Effort*
Project Role:	*Post-Doc*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*6*
Contribution to Project:	*Dr. Ramchandani has performed all miRNA manipulations and has worked with Weill Cornell collaborators to optimize gold nanoparticles for miRNA packaging and in vivo delivery.*
Funding Support:	

Name:	*Sharrell Lee (Technician - WCM) – 15% effort*
Project Role:	*Technician*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1.8*
Contribution to Project:	*Ms. Lee has assisted Dr. Ramchandani in the in vivo work.*
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?

No

What other organizations were involved as partners?

None

8. **SPECIAL REPORTING REQUIREMENTS**
   Nothing to report

9. **APPENDICES:**
   Nothing to report