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TITLE: Targeting Neutrophil Protease-Mediated Degradation of Tsp-1 to Induce Metastatic Dormancy

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14. ABSTRACT

Background.
External pre-existing inflammation in the lungs is linked to increased incidence of metastasis. Inflammation –mediated by bacterial infection or cigarette smoke enhanced pulmonary metastasis from breast cancer in humans and mice. Similarly, autoimmune arthritis, characterized by increased recruitment of inflammatory neutrophils and macrophages in the lungs was associated with increased breast cancer metastasis to the lungs. Despite this compelling link between inflammation and metastasis, the mechanisms by which inflammation contributes to tumor outgrowth in distant metastatic organs have remained underexplored. We believe that targeting inflammation-mediated metastasis has tremendous potential in the treatment of high-risk breast cancer patients.
Overarching challenges. Breast cancer affects more than 1.7 million individuals a year worldwide, with approximately 500,000 deaths. Importantly, >90% of this mortality is a consequence of metastatic disease that is resistant to adjuvant therapies. Despite this clinical significance, there is a conspicuous lack of a single FDA approved molecularly targeted anti-metastatic therapy. Hence, there is an urgent medical need to develop new targeted anti-metastatic therapeutic approaches. However, a lack of mechanistic understanding by which tumor cell colonize and outgrow in distant metastatic organs, has been a major impediment to the development of an effective anti-metastatic therapy.
Hypothesis /Objective. We hypothesize that intervention against inflammation-driven neutrophil elastase (NE)/Cathepsin G (CG)-Thrombospondin-1 (Tsp-1) axis can be developed into an anti-metastatic therapy in breast cancer. Our objectives are: 1) to establish that the neutrophil NE/CG-Tsp-1 axis is the dominant pathway in inflammation-mediated metastasis, 2) to determine the molecular mechanisms by which neutrophil CG/NE-Tsp-1 axis promotes metastasis, 3) to show that NE/CG-Tsp-1 axis modulates Tsp-1-mediated metastatic dormancy, 4) to assess whether pharmacological inhibition of CG/NE can be used to inhibit metastasis, and 5) to determine if induction of Tsp-1 expression in the lung microenvironment with a novel DWLPK peptide constitutes an anti-metastatic approach. Our overall goal is to develop a mechanism-guided intervention against inflammation-driven breast cancer metastasis.
Specific Aims. 1) To determine the role of neutrophil NE/CG-Tsp-1 axis in breast cancer metastasis to the lung; 2) To determine if pharmacological inhibition of NE and CG can be used to inhibit metastasis, and 3) To determine if ectopic induction of Tsp-1 expression in the lung microenvironment blocks NE/CG-mediated metastasis.
Study Design. We have recently demonstrated that external inflammation in the lungs is associated with increased incidence of metastasis. We discovered a novel mechanism, whereby abundant neutrophils recruited in the inflamed lungs degranulate their azurophilic granules to release two key serine proteases, CG and NE. These proteases specifically target the tumor suppressor Tsp-1, for proteolysis, to generate tumor-promoting microenvironments. Using a combination of genetic and pharmacological approaches, we will determine the mechanistic role and therapeutic potential of CG/NE-Tsp-1 axis in inflammation-mediated breast cancer metastasis.
Innovation. This proposal addresses the critical and unique link between pre-existing inflammation in the lungs and increased incidence of metastasis from breast cancer. A variety of mouse genetic models, together with compartment-specific gene knockout strategies will be employed. In parallel, pharmacological approaches will be used to complement the genetic strategies, and to provide feasibility for clinical translation. This study emphasizes that therapy should be targeted against the reprogrammed host microenvironment, which contributes to, and supports, the e growth and survival of disseminated tumor cells
Impact. We expect to unravel mechanistic and therapeutic insights and generate unique translational opportunities and may lead to the design of future clinical trials for high-risk breast cancer patients that exhibit inflammation (Cigarette smoke, COPD/emphysema related). Notably, the dual NE/CG protease inhibitor Sivelestat is available and is currently being used in Phase III clinical trials of acute lung injury with systemic inflammatory response syndrome. We expect that findings from our studies will support the potential for repurposing Sivelestat as a dual protease antagonist in the treatment of metastasis in breast cancer patients with lung inflammation. Similarly, induction of Tsp-1 expression with a novel DWLPK peptide drug either alone or in combination with Sivelestat has tremendous potential for designing future clinical trials for high-risk breast cancer patients.

15. SUBJECT TERMS
Triple negative breast cancer, metastasis, lipopolysaccharide, thrombospondin 1, cathepsin G, bone marrow transplantation, neutrophil elastase, sivelestat

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Table of Contents

Page

1. INTRODUCTION:

We hypothesize that intervention against inflammation-driven NE/CG- Tsp-1 axis can be developed into an anti-metastatic therapy in breast cancer. Using a combination of genetic and pharmacological approaches, we propose to achieve the following objectives; 1) to establish that the neutrophil NE/CG-Tsp-1 axis is the dominant pathway in inflammation-mediated metastasis, 2) to determine the molecular mechanisms by which neutrophil CG/NE-Tsp-1 axis promotes metastasis, 3) to show that NE/CG-Tsp-1 axis modulates Tsp-1-mediated metastatic dormancy, 4) to assess whether pharmacological inhibition of CG/NE with Sivelestat can be used to inhibit metastasis, and 5) to determine if induction of Tsp-1 expression in the lung microenvironment with a novel DWLPK peptide constitutes an anti-metastatic approach.

This project addresses BCRP overarching challenges of revolutionizing treatment regimens by replacing interventions that have life-threatening toxicities with ones that are safe and effective; and for advancing the field towards the elimination of mortality associated with metastasis in high-risk breast cancer patients. It also addresses metastatic dormancy, and progression of breast cancer to life threatening metastasis. In summary, we anticipate that the proposed studies will lead to exciting and novel findings that have the potential to impact inflammation-mediated metastasis in breast cancer.

2. KEYWORDS:

breast cancer, metastasis, Thrombospondin 1, neutrophil, inflammation, metastases

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Aim1: To determine the role of neutrophil NE/CG-Tsp-1 axis in breast cancer metastasis to the lung.

Major Task 1: Determine if the metastasis-suppressive phenotype in NE-/-CG-/- mice can be rescued in Tsp-1-/- mice.

Subtask 1: Generate cohorts of WT, Tsp-1-/- and TKO BMT mice
Subtask 2: Generate LPS-mediated inflammation in WT, Tsp-1-/- and NE-/- CG-/- Tsp-1-/- mice, administer tumor cells (EO771& PyMT).
Subtask 3: Resect primary tumors and evaluate metastasis in lungs. Characterize phenotypes. Animals: (n=15 per cohort × 3 cohorts × 2 tumor models X repeat expt): 180 mice

Major Task 2: Determine whether loss of NE/CG-Tsp-1axis impacts metastasis by regulating angiogenesis, or proliferation/apoptosis of tumor cells via Tsp-1 receptor CD36.

Subtask 1: Generate shRNA-mediated loss of CD36 expression in tumor cells
Subtask 2: Administer WT and shRNA- tumor cells into WT, Tsp-1-/- and NE-/-CG-/- BMT mice. Animals: (n=15 per cohort × 3 cohorts × 2 tumor models X 2shRNA X repeat): 360 mice

Major Task 3: Determine if Tsp-1 in the lung modulates metastatic dormancy in WT, Tsp-1-/- and NE-/- CG-/- BMT mice. (n=15 per cohort × 3 cohorts × 2 tumor models X repeat): 180 mice

Milestone(s) Achieved: Generation of TKO mice, establish role of NE/CG-Tsp-1 axis. CD36 receptor in inflammation-mediated metastasis, Metastatic dormancy

Aim 2: To determine if pharmacological inhibition of NE and CG can be used to inhibit metastasis.

Major Task 1: Pharmacological inhibition of NE/CG with Sivelestat in WT, Tsp-1-/- NE-/-CG-/- and TKO BMT mice. (n=15 per cohort × 4 cohorts × 2 tumor models X repeat): 240 mice

Major Task 2: Efficacy of Tsp-1-mimetic peptide in inhibiting angiogenesis Tsp-1 deficient lungs. ABT-510 peptide in inhibiting angiogenesis. (n=15 per cohort × 4 cohorts × 2 tumor models X repeat): 240 mice

Milestone(s) Achieved: Determine pharmacological efficacy of Sivelestat and ABT-510 in inflammation-mediated metastasis.

Aim 3: To determine if induction of Tsp-1 expression in the lung microenvironment blocks NE/CG-mediated metastasis.

Major Task 1: Evaluate efficacy of DWLPK peptide in WT, Tsp-1-/- and NE-/- CG-/- BMT mice (n=15 cohort × 3 cohorts × 2 tumor models X repeat): 180 mice+ 30 (scramble controls)= 210 mice
Combine DWLPK with Sivelestat in WT LPS challenged cohorts only (n=15 per cohort × 3 cohorts × 2 tumor models X repeat): 360 mice

Milestone(s) Achieved: Demonstrate efficacy of DWLPK and combined DWLPK and sivelestat in metastasis.

What was accomplished under these goals?

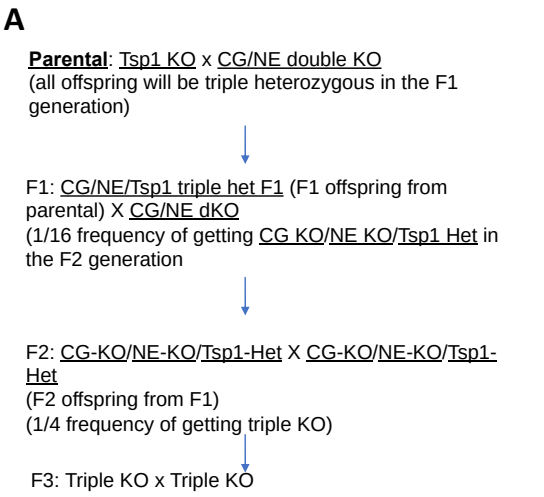
For this reporting period, we are reporting progress for the following:

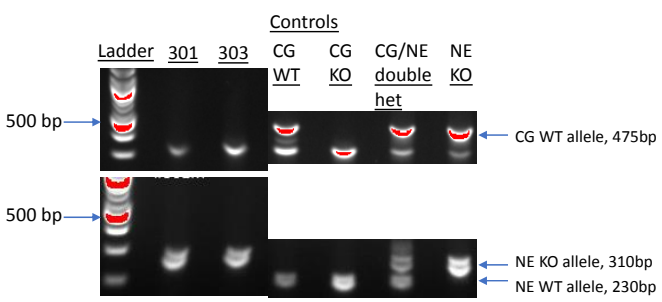
Aim1: To determine the role of neutrophil NE/CG-Tsp-1 axis in breast cancer metastasis to the lung.

Major Task 1: Determine if the metastasis-suppressive phenotype in NE-/-CG-/- mice can be rescued in Tsp-1-/- mice.

Subtask 1.1 Generate TKO (CG-/-;NE-/-;Tsp-1-/-)BMT mice

To generate the triple knockout (CG-/-NE-/-Tsp-1-/-) mice we used the breeding strategy shown in Fig. 1A. We had generated TKO heterozygous mice and were expecting to have the homozygous TKO from breeding these mice. However, the average litter size for the F2 generation breeders (**Fig. 1A**) was small, ranging from 4-8 pups. Also, given the low expected frequency of an F2 second generation breeder (1/8) as shown in Fig. 1a, the odds of getting a TKO are even lower. Ultimately, to achieve a better breeding strategy and thus better allele frequency, at best we plan to generate crosses of TKO x TKO (all offspring TKO), or at least TKO X CG-/-NE-/-/Tsp1+/- (1/2 offspring TKO), which would be F3 generation. However, to generate a sufficient cohort size, multiple breeders of these types are required for experiments, which may require at least 2-3 litters from the F2 generation. As a result, we have reached a standstill at the F2 generation of obtaining TKO for line expansion to yield sufficient animals for experiments. Another bottleneck is that from F2 generation, inspite of getting several litter droppings, we only obtained 2 TKO pups, both of which were males, and thus we were unable to set the F3 breeder. Therefore, while the breeding is ongoing, we will use Sivelestat to generate NE' CG loss of function in a Tsp-1-/- mice.





C

T556325

Tsp1 KO Strain Data

Neomycin

Well	Sample Name	Signal	Final Result
A1	301	3.877	+
B1	302	3.948	+
C1	303	7.896	+

	Tsp1 KO	Neomycin	Thbs1 KO	Thbs1 WT
Wellplate: T556325				
A1	301	+	+	+
B1	302	+	-	+
C1	303	+	+	+

Figure 1

A) Breeding strategy to generate CG/NE/Tsp1 triple KO mice, B) Cathepsin (CG) and Neutrophil Elastase (NE) PCR genotyping (CG WT allele is 475bp, absence of 475bp allele indicates CG KO, NE WT allele is 230bp and NE KO allele is 310bp and C) Transnetyx raw data for Tsp1 (neomycin), and genotyping data (the signal is calculated by normalizing the raw data from Tsp1 probe to a housekeeping gene for Tsp1). 301 (male) and 303 (female) are CG KO/NE KO/Tsp1 Het, which will subsequently be crossed to each other to generate triple knockout (frequency 1:4).

<u>Subtask 2:</u> Generate LPS-mediated inflammation in WT, Tsp-1-/- and NE-/- CG-/- Tsp-1-/- mice, administer tumor cells.

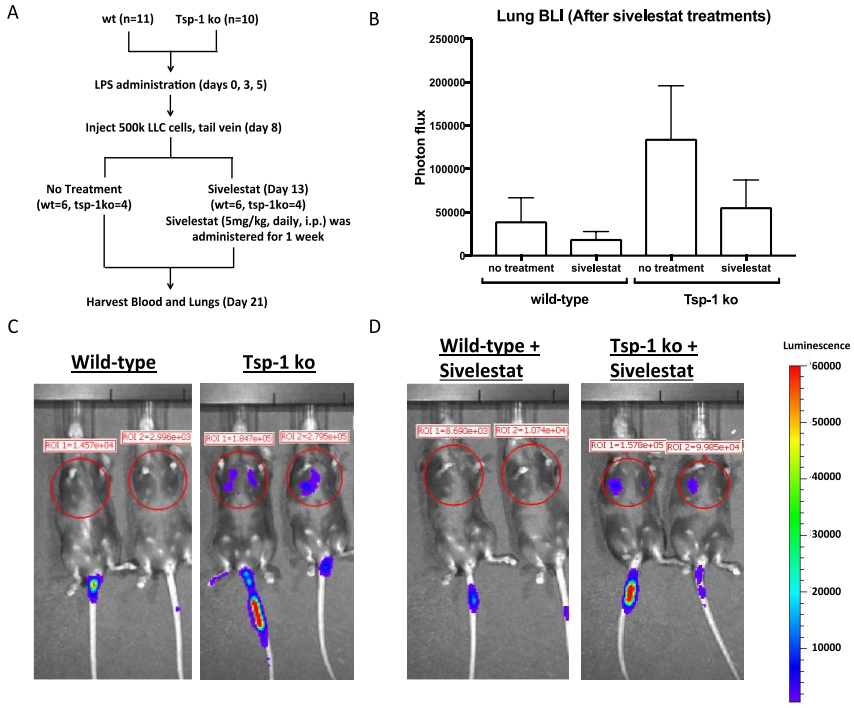


Figure 2. Tsp-1 KO enhances lung metastases. (A) Schematic of experimental design. (B) Lung BLIs after LLC cells were injected via tail vein, but before any sivelestat treatments. (C) Lung metastasis were higher in tsp-1 ko mice, and inhibition of NE/CG via sivelestat treatments was able to reduce lung tumor burden. (D-E) Representative BLI images of the four cohorts.

The goal was to demonstrate if metastasis-suppressive phenotype in $NE^{-/-}CG^{-/-}$ mice can be rescued in $Tsp\text{-}1^{-/-}$ mice. Given that we were unable to generate triple KO mice, we treated Tsp-1 KO mice with Sivelestat, an inhibitor of NE and CG to achieve deficiency of NE, CG and Tsp-1. The experimental design is illustrated in **Fig. 2A**.

To generate local lung inflammation, LPS was administered intranasally in a 50 µl volume at a concentration of 0.25 mg/ml every three days in wild-type, Tsp-1 KO and Tsp-1KO treated with Sivelestat. As expected in the absence of Sivelestat treatment, Tsp-1 KO mice showed increased metastasis compared to wild-type mice **(Fig. 2B-C)**. Importantly, compared to wild to mice sivelestat was unable reduce metastais Tsp-1 KO mice **(Fig. 2B, D)** indicative of a functional NE/CG-Tsp-1 axis in inflammation-dependent metastasis. However, unexpectedly, metastatic burden in Tsp-1 KO group treated with sivelestat was lower compared to Tsp-1 KO mice **(Fig. 2B, D)**. These findings suggest that sivelestat may also have an effect independent of the NE/CG-Tsp-1 axis in regulating metastasis.

We have begun to explore this by evaluating the effect of Sivelestat on the tumor microenvironment. First, we performed a comprehensive analysis of bone marrow-derived immune cell subsets in the peripheral blood. Blood samples were collected via retro-orbital sinus puncture into anticoagulant EDTA tubes. PBMCs were extracted using ficoll. Cell pellets were washed with FACS buffer by centrifuging at 1500 RPM for 5 minutes. For surface stains, samples were blocked with anti-mouse CD16/32 for 15 minutes at room temperature, incubated with primary antibodies for 45 minutes in dark on ice, washed with FACS buffer, fixed with 1% formaldehyde for 30 minutes at room temperature in dark, washed with FACS buffer, resuspended in FACS buffer and stored at 4ºC until analysis, which was performed within 24 hours of staining. For distinguishing different myeloid-derived cells, single cell suspensions were stained with CD45, CD11b, CD11c, F4/80, Ly-6G, Ly-6C, MHC-II, CD3, B220, CD4, and CD8a. All antibodies were obtained from Biolegend and used at a

dilution of 1:100. We did not see any differences in any cell type **(Fig. 3-4)** except neutrophils **(Fig. 3A)**, which were higher in blood in Tsp-1 KO mice without sivelestat treatment.

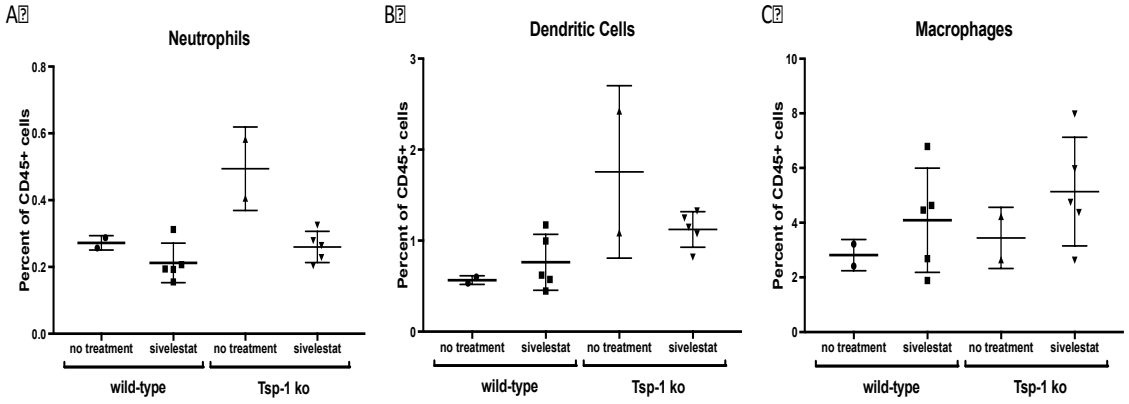


Figure 3. Sivelestat treatments may impact Neutrophils in blood. Impact of sivelestat treatments on myeloid population in blood was analyzed for the four cohorts. No change was observed in the number of dendritic cells (B) or macrophages (C) among the four groups. However, neutrophils (A) seem to be higher in Tsp-1 ko mice over wt or sivelestat-treated groups.

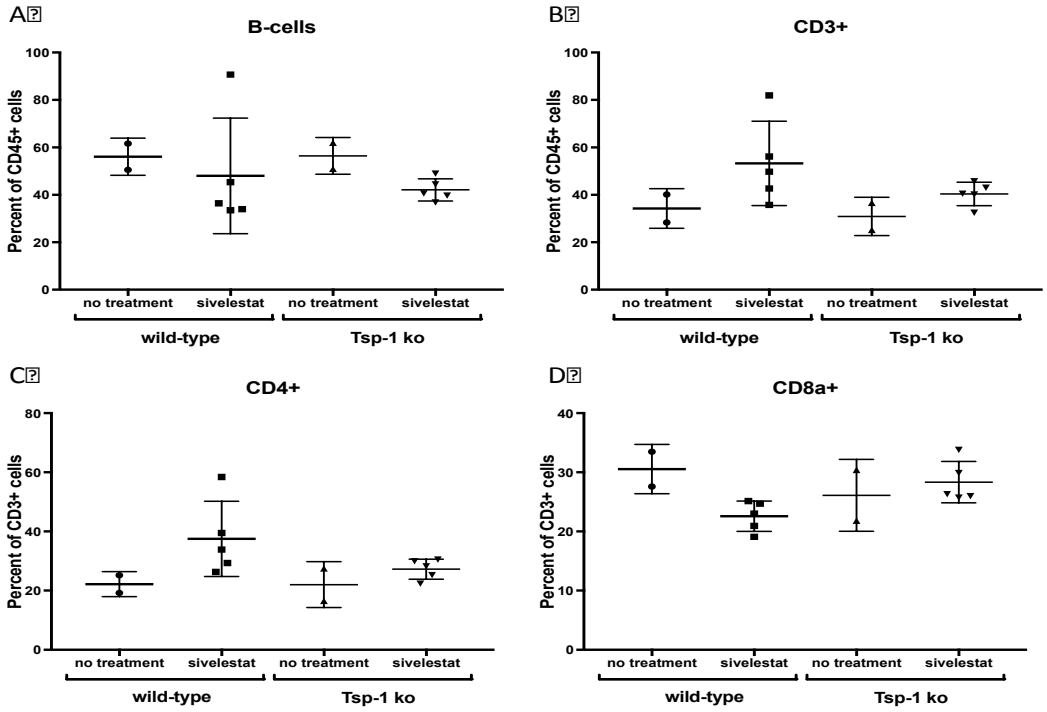


Figure 4. Sivelestat treatment does not impact B- or T-cells. Sivelestat treatment did not impact the total number of B-cells (A), CD3+ (B), CD4+ (C), or CD8+ (D) cells.

<u>Subtask 3:</u> Resect primary tumors and evaluate metastasis in lungs. Characterize phenotypes.
Animals: (n=15 per cohort × 3 cohorts × 2 tumor models X repeat expt): 180 mice
We used an immunocompetent EO771 primary TNBC model in C5BL/6 mice (Wt or Tsp-1 KO, n = 12/group). 100k tumor cells in HBSS were injected into the fourth mammary fat pad of mice. The tumors were allowed to grow until they reached 1 cm^3, at which point they were resected, and mice were monitored for lung BLIs until they died of lung tumor burden to generate a survival curve. We did not see any significant difference in lung metastasis of Tsp-1 KO mice compared to Wt cohort. Furthermore, the overall survival for the two groups remained unchanged **(Fig. 5)**. There is a possibility that the primary breast tumors may have systemically reprogrammed the metastatic lungs, which may have neutralized the metastasis promoting effect of Tsp-1 loss. To exclude this possibility, and to evaluate the impact of Tsp-1 exclusively in the metastatic lung without confounding effects of the primary tumors, we will explore the experimental metastasis model with EO771 cells in the presence and absence of LPS induced inflammation.

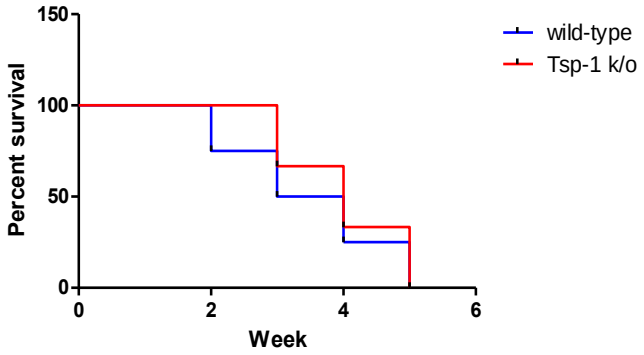


Figure 5. Survival of Tsp-1 ko mice over wl group was not significantly impacted in EO771 primary breast tumor model. X-axis depicts weeks after primary tumor resection.

Task 3.1. Determine if Tsp-1 in the lung modulates metastatic dormancy.
EO771 TNBC cells expressing mCherry and luciferase reporters were administered in the mammary glands. Tumor growth was monitored and primary tumors were resected. Single disseminated cells in the metastatic lungs (mcherry+) expressed dormancy markers including p38, p21 and low pERK (**Fig. 6**) as low pERK/p38 signaling ratio is associated with induction of both quiescence and survival signaling, which leads to dormancy (Aguirre-Ghiso et al., 2013).

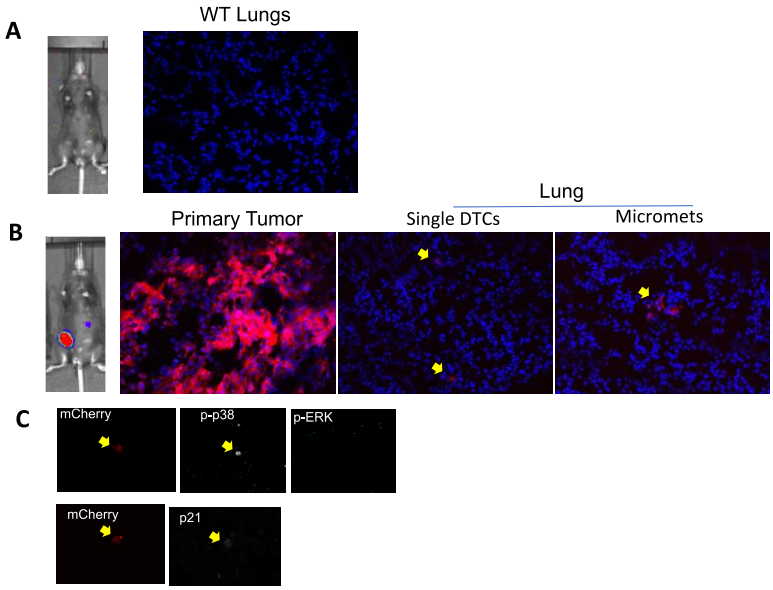


Fig. 6 Metastatic dormancy from EO771 primary tumors.
A) Lungs from a wt type non tumor bearing mice; B) Primary tumors and lungs form mice implanted with mcherry+ luc+ E0771 cells in the mammary gland.
Yellow arrows indicated disseminated tumor cells in lungs
C) Single disseminated mcherry+ cells in lungs exhibit dormancy phenotypes including High xpression of p-p38, p21 and low expression of p-ERK.

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?
In Year 2, Dr. Mittal has given invited seminars (CSBC Annual Meeting – Broad Institute, Cambridge, MA Oct 2017, TEMTA Meeting MD Anderson Cancer Center, Houston, TX Dec 2017, Sylvester Cancer Center Miami, FL Dec 2017, PSOC Annual Retreat Ithaca, NY Jan 2018, IUMB Symposium Seoul, Korea June 2018, CBSC Symposium, Washington DC Sept 2018) on this topic.

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

1) We will continue to determine mechanisms by which NE/CG-Tsp1 axis mediates metastasis.

2) Explore the experimental metastasis model with EO771 cells in the presence and absence of LPS induced inflammation.

3) Generate CD36 knockout in tumor cells and determine impact of Tsp-1 in metastasis.

4) If Tsp-1 in the lung modulates metastatic dormancy in WT, Tsp-1-/- and NE-/- CG-/- BMT mice.

5) Determine if DWLPK mediated induction of Tsp-1 resulting in metastasis suppression is rescued with sivelstat.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?
In year 1, we have focused on generating reagents and strategies for the planned in vivo studies. In year 2, we began in vivo experiments to interrogate the metastatic cascade as well as metastatic dormancy. In year 3, we hope to overcome some of our KO mouse breeding problems and expand on the in vivo work. We anticipate that the proposed studies will lead to novel findings that have the potential to finally impact inflammation-mediated metastasis in breast cancer.

What was the impact on other disciplines?
Progress in elucidating inflammation-mediated metastasis pathways is likely to attract many investigators across disciplines in breast cancer research and result in rapid advancements towards finding a potential therapy against metastatic breast cancer.

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:

Aim 1, Subtask 1.1: Generate cohorts of WT, Tsp-1-/- and TKO BMT mice
As we have experienced problems with the solubility of the DWLPK peptide, better strategies for the DWLPK peptide solubility were created and optimized.

Aim 1, Subtask 1.3: Resect primary tumors and evaluate metastasis in lungs. Characterize phenotypes.
We analyzed the role of EO771 metastasis from primary tumor site in wt versus Tsp-1-/-. We did not see any significant changes. This may be because primary breast tumors may be systemincally reprogramming the premetastatic sites in the lungs beyond just inflammation. Therefore to bypass this, and obtain the most direct evidence, we plan to use EO771 experimental metastasis model after inducing inflammation in lungs by LPS.

Aim 2, Task 1.2: Pharmacological inhibition of NE/CG with Sivelestat in WT, Tsp-1-/- NE-/-CG-/- and TKO BMT mice.
As expected in the absence of Sivelestat treatment, Tsp-1 KO mice showed increased metastasis compared to wild-type mice. Importantly, compared to wild to mice sivelestat was unable to reduce metastais Tsp-1 KO mice indicative of a functional NE/CG-Tsp-1 axis in inflammation-dependent metastasis. However, unexpectedly, metastatic burden in Tsp-1 KO group treated with sivelestat was lower compared to Tsp-1 KO mice. This finding suggests that sivelestat may also have an effect independent of the NE/CG-Tsp-1 axis in regulating metastasis.

6. PRODUCTS:

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Vivek Mittal (PD/PI) – 15% Effort*
Project Role:	*PD/PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1.8*
Contribution to Project:	*Dr. Mittal led the project and oversaw all aspects of the strategy for planning experiments, etc.*
Funding Support:	

Name:	*Divya Ramchandani, PhD (Post-Doc) – 33% Effort*
Project Role:	*Post-Doc*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*4*
Contribution to Project:	*Dr. Ramchandani has performed all neutrophil degranulation assays, flow cytometry, and in vivo experiments*
Funding Support:	

Name:	*Sharrell Lee (Technician) – 50% effort*
Project Role:	*Technician*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*6*
Contribution to Project:	*Ms. Lee has assisted Dr. Ramchandani on the in vitro work, flow cytometry, animal handling. Most importantly, she has worked on breeding the triple knockout mice. Ms. Lee has helped design and manage the animal experiments.*
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