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14. ABSTRACT

Over 200,000 new cases of invasive breast cancer are diagnosed in the United States each year and account for approximately 40,000 deaths. From a treatment perspective, breast cancer is a paradigm for individualized medicine with two personalized therapies in use; endocrine therapy for hormone receptor positive patients and HER2-targeted agents such as trastuzumab for HER2+ patients. However, patients with TNBC (basal-like subtype lacking all three receptors (ER, PR and Her2/neu) are refractory to these therapies. Surgical resection and standard chemotherapy regimens remain the only therapeutic options for women with TNBC, and these treatments usually fail resulting in an aggressive metastatic relapse and short overall survival. Therefore, there is an urgent need to develop new-targeted therapeutic approaches. This proposal provides a mechanism-based approach, which promises to impact the treatment of TNBC, a subtype of highly metastatic breast cancer that confers the worst outcome.

We have identified miR-708 as a potential "metastasis suppressor" in breast cancer. miR-708 targets neuronatin to decrease intracellular calcium level, which inactivates ERK/FAK pathways to impair cell migration and metastases. Analysis of miR-708 upstream regions showed enrichment of PRC2 which was associated with elevated H3-K27me3 levels. We hypothesize that PRC2-induced H3-K27me3 silences miR-708 in metastasis. Significantly, systemic delivery of synthetic miR-708 blocked TNBC metastases, providing a rationale for developing miR-708 as a novel therapeutic agent against metastatic breast cancer. Our objective is to dissect the epigenetic regulation of miR-708, so that epigenetic therapies can be considered for metastatic breast cancer, and evaluate the therapeutic efficacy of synthetic miR-708.

Dissecting the epigenetic regulation of miR-708 will generate translational opportunities for patients with TNBC. For example, insights into the role of PRC2 to directly mediate miR-708 silencing will allow the evaluation of epigenetic therapy in metastatic breast cancer. Our demonstration that restoration of miR-708 attenuates metastasis following metastatic colonization suggests the possibility of directly using miR-708 as a therapeutic modality. Of note, treatment with miR-708 is likely to target the more aggressive metastatic breast cancer cells that lack miR-708 and not affect normal tissues that maintain expression of miR-708.

In summary, the therapeutic potential of miR-708 may lead to the design of future clinical trials for the treatment of extraordinarily high-risk breast cancer patients whose tumor has undergone metastatic dissemination (Stage 4, NED, TNBC). Given the strong preclinical data that would emerge from this grant, we expect that with a rapid clinical translation the approximate time for these potential therapeutics to move from bench to bedside will be about 5-10 years.

15. SUBJECT TERMS

Breast cancer, Metastasis, miR-708, Epigenetic, Nanoparticle, Targeted therapy

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1. INTRODUCTION:

This proposal aims to dissect the epigenetic regulation of miR-708 by EZH2, so that epigenetic therapies can be considered for metastatic breast cancer. In addition, it will also evaluate the therapeutic efficacy of synthetic miR-708 in the treatment of breast cancer metastasis. The research plan is based on the hypothesis that PRC2-induced H3-K27me3 silences miR-708 in metastasis. The project's specific aims are (1) to determine the mechanism by which PRC2 complex regulates miR-708 in breast cancer metastasis and (2) to assess the therapeutic potential of miR-708 against metastatic triple-negative (TN) breast cancer using nanoparticle delivery system. A major goal of this study is that it aims to provide a targeted therapy for TN breast cancer, which has no current targeted therapy.

2. KEYWORDS:

Breast cancer, Metastasis, miR-708, Epigenetic, Nanoparticle, Targeted therapy.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Aim1:

Major Goal 1: PRC2 Knockdown and evaluate miR-708, Nnat, migration in vitro

Subtask 1: Perform shRNA-mediated suppression of Suz12, Ezh2 in various breast cell lines.

Subtask 2: Evaluate consequence on miR-708 and Nnat levels, cell migration.

Subtask 3: Perform shRNA-mediated suppression of Suz12 in various breast cancer cell lines and MMTV cell lines and evaluate consequence on metastasis in vivo.

Major Goal 2: PRC2 Knockdown and evaluate metastasis in vivo

Subtask 1: Pharmacological inhibition of EZH2. Perform suppression of EZH2 HMT in MDA-23-LM2, MDA-436 and MDA-361, MCF7 and MMTV-PyMT cell lines by GSK126 and Evaluate consequence on metastasis in vivo with MDA-MB231-LM2 and MMTV-PyMT.

Subtask 2: Measure levels of miR-708, Nnat, cell migration and invasion

Subtask 3: Determine if PRC2 blockade impacts metastasis through miR-708. (Perform miR-708 ablation either using sponge or CRISPR method in MDA cells) and determine if pharmacological blockade of EZH2 is incapable of metastasis suppression

Aim 2:

Major Goal 1: Assess the therapeutic potential of miR-708 against metastatic TNBC breast cancer

Subtask 1: MDS for delivery of miR-708 to prevent metastases derived from orthotopic tumors.

Subtask 2: MDS for delivery of miR-708 to treat heterotransplanted patient breast cancer.

Milestone(s) to be achieved: demonstrate that miR708 can block TNBC mets in both models

What was accomplished under these goals?

For the final report, we are summarizing major progress made in all the aims, including the progress made during the no cost extension period.

Aim1

Aim1 Major Goal 1: PRC2 Knockdown and evaluate miR-708, Nnat, migration in vitro

Subtask 1: Perform shRNA suppression of Suz12, Ezh2 in various breast cell lines.

We had showed that EZH2 levels are elevated in TNBC cells with high metastatic potential compared to non-metastatic counterparts, and miR-708 expression levels show an inverse correlation to EZH2 (**Fig. 1A**). shRNA-mediated knockdown of EZH2 resulted in cell migration/invasion defects (data not shown). To demonstrate that the PRC2 is directly responsible for silencing miR-708, shRNA-mediated knockdown EZH2 (>2 shRNAs), showed that EZH2 knockdown restores miR-708 expression in MDA-MB-231-LM2 cells (**Fig. 1B**).

To determine how PRC2 regulates miR-708 expression, in Aim 1 we had proposed two approaches to target PRC2. The first approach included shRNA-mediated knockdown of PRC2 subunits SUZ12 or EZH2, and the second approach involved pharmacological inhibition of EZH2 methyltransferase.

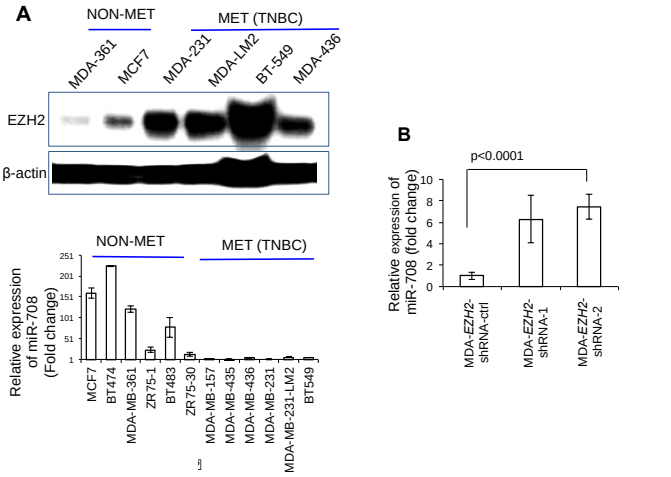

Fig. 1. EZH2-miR-708 axis in breast cancer metastasis. (A) EZH2 and miR-708 expression in TNBC breast cancer cell lines (upper and lower panels respectively), **(B)** Conditional knockdown of EZH2 with EZH2-shRNA restores miR-708 expression.

shRNA suppression of Suz12 and EZH2 performed in MDA-MB231-LM2 cells, showed that EZH2 knockdown cells cells survived in culture only for a short culture period, a phenotype that was opposed to that observed with specific inhibition of EZH2 catalytic function. This is due to the destabilization of the PRC2 complex, as a result, specific canonical contributions EZH2 histone methyltransferase (HMT) activity on ensuing phenotypes cannot be deciphered. Therefore, in lieu of performing EZH2 knockdown, we had advocated the use of EZH2 histone methyl transferase inhibitor for long tern *in vitro* experiments (migration, invasion) and *in vivo* experiments (tumor growth and metastasis).

To achieve specific and direct inhibition of the EZH2 catalytic activity, we used GSK126, a specific pharmacological inhibitor of EZH2 HMT (McCabe et al., 2012). Dose optimization studies showed that GSK at 2-5μM effectively inhibits EZH2 HMT in vitro (data not shown). Importantly, GSK126 specifically blocked EZH2-mediated H3K27 trimethylation (and not the related H3K4me trimethylation), and did not affect EZH2 protein (**Fig. 2A**) indicating marked specificity. Intact EZH2 levels also indicated that the PRC2 complex was not degraded.

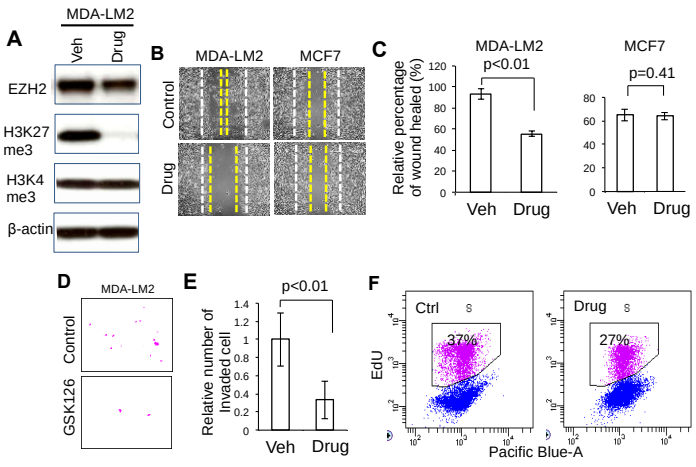

Fig. 2. EZH2 blockade impacts cell migration and invasion. (A) Metastatic breast cancer cells treated with vehicle (Veh) or EZH2 HMT inhibitor (GSK126, 5uM) for 3 days. Metastatic breast cancer cell MDA-LM2 or non-metastastatic breast cancer MCF7 treated with vehicle (Veh) or EZH2 HMT inhibitor (Drug, 5uM) for 3 days. Effect of the drug on cell migration **(A-B)**, invasion through a matrigel coated transwell **(C-D),** and proliferation.

GSK126 suppressed migration (**Fig. 2B-C**), and invasion of metastatic breast cancer cells (**Fig. 2E)** without significant effects on proliferation (**Fig. 2F**).

However, we soon realized that it would be necessary to obtain genetic validation of the pharmacological approach. Therefore, we used a innovative approach to engineer a novel mutation using CRISPR/Cas9 in the catalytic SET domain of EZH2 (see below).

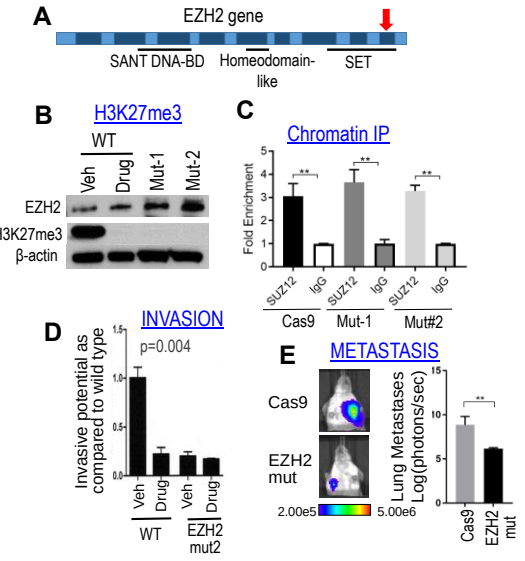

Fig. 3. (A) CRISPR-Cas9 used to generate a catalytically inactive EZH2 SET domain mutant. **(B)** EZH2 mutant show reduced H27me3. **(C)** ChIP for Suz12 showing EZH2 mutant is part of intact PRC2. **(D)** EZH2 mutant exhibits loss of invasion, **(E)** EZH2 mutant impairs lung mets from primary orthotopic tumors. ** denotes significant (p<0.05)

Catalytically inactive EZH2 mutant exhibits phenotypes similar to pharmacological inhibition. Given that genetic knockout of EZH2 subunit leads to the degradation of PRC2 complex, we used CRISPR/Cas9 to engineer a point mutation in the catalytic core of the SET-domain (**Fig. 3A**). Notably, the mutation inactivated the methyltransferase activity without degrading EZH2 and other subunits of PRC2 as determined Western blotting and ChIP, indicative of an intact PRC2 (**Fig. 3B-C**). EZH2 mutant showed impaired invasion (**Fig. 3D**), did not impact primary tumor growth (data not shown), but reduced lung metastasis (**Fig. 3E**). This unique EZH2 mutant will serve as a powerful genetic tool to evaluate EZH2 catalytic functions, and will be used to complement the pharmacological approach

Subtask 2: Evaluate consequence on miR-708 and Nnat levels, cell migration.

Pharmacological inhibition of EZH2 restores miR-708 expression and inhibits Nnat: Consistent with the genetic analysis that EZH2 knockdown restores miR-708 expression (**Fig. 1B**), pharmacological inhibition of EZH2 by administration of GSK126 restored miR-708 expression in metastatic TNBC cells and not in non-metastatic cells, which express high steady state levels of miR-708 (**Fig. 4A**). Importantly, restoration of miR-708 expression was associated with a decrease in the levels of Nnat (miR-708 target) (**Fig. 4B**).

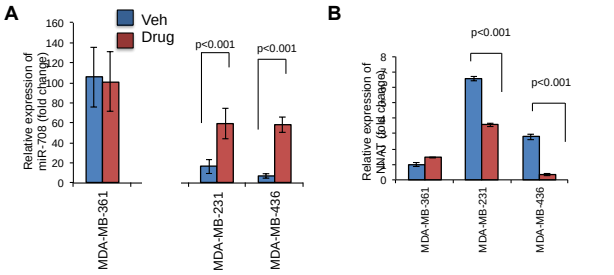

Fig. 4. EZH2 inhibitor restores miR-708 expression and inhibits Nnat. **(A)** 5 μM GSK126 restores miR-708 expression in metastatic cell lines, MDA-MB-231 and MDA-MB-436 at day 10. Non-metasatic MDA-361 (EZh2 low) expresses high levels of miR-708 and remains unaffected by GSK126. (**C**) Restoration of miR-708 results in concomitant downregulation of its target Nnat.

Subtask 3: Perform shRNA-mediated suppression of Suz12 in various breast cancer cell lines and MMTV cell lines and evaluate consequence on metastasis in vivo.

As stated above, in lieu of performing EZH2 knockdown, we used EZH2 histone methyl transferase inhibitor GSK126 for in vivo experiments.
To determine the impact of EZH2-miR-708 axis in breast cancer metastasis to the lungs, we generated orthotopic tumors in the mammary glands of SCID mice with 1×10^6 viable MDA-LM2 cells (basal subtype, ER^-, PR^-, $HER2^-$)(Neve et al., 2006), stably expressing luciferase and GFP transgenes. As shown in the schematic (**Fig. 5**), primary tumors were allowed to grow for 4 weeks (n=10/group) and then resected as described (Ryu et al., 2013). We have chosen this particular window for resection, as there are no detectable metastases in the lungs. Next, mice (10 mice/group)

were treated with GSK126 (150 mg/kg, i.p. twice a week) or vehicle control (20% captisol) for two weeks and monitored for metastases (see treatment regimen schema, **Fig. 5**). Significant reduction in metastasis was observed in drug treated group compared to controls as determined by BLI **(Fig. 5, Group 1**). This phenotype was reproduced in an independent group of mice (**Fig. 5**, **Group 2**). From the clinical perspective, this treatment schema mirrors an adjuvant therapy approach, with the expectation that such a treatment plan may prevent or delay the onset of distant metastases.

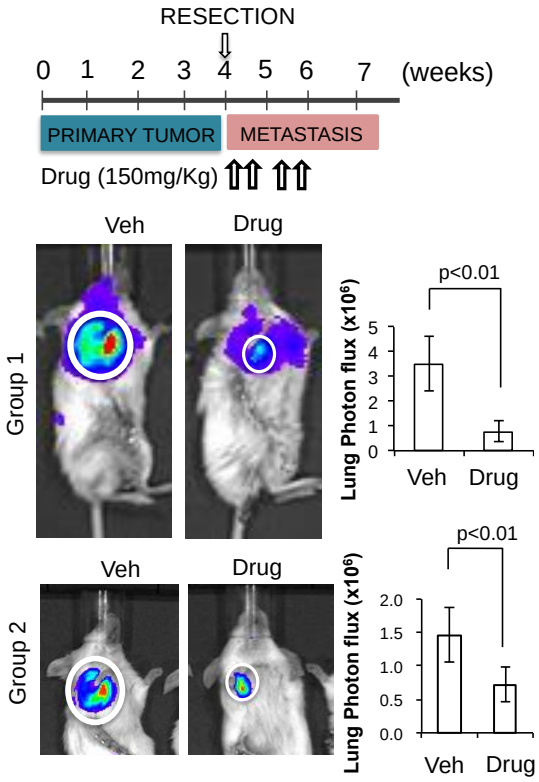

Fig. 5. EZH2 inhibition impairs metastasis. Schematic of adjuvant treatment strategy with 150 mg/kg GSK126 drug following surgical resection of primary breast tumor in mammary gland. Impact on lung metastases in two independent group of mice.

Aim 1 Major Goal 2: PRC2 Knockdown and evaluate metastasis in vivo

Subtask 1: Pharmacological inhibition of EZH2 in various TNBC cell lines and evaluate consequence on metastasis in vivo.

We collected a number of metastatic murine and human breast cancer cell lines to evaluate EZH2 HMT inhibition. These cell lines include a C57BL/6-mouse- derived line EO771, which give rise to orthotopic adenocarcinomas with core basal phenotype, defined as triple-negative tumors expressing either CK5/6 and/or EGFR. Notably, GSK126 efficiently blocked EZH2-mediated H3K27 trimethylation in a dose dependent manner and impaired invasion across a matrix coated transwell membrane (**Fig. 6**). We also used the Met 1 cell line, which is derived from MMTV-PyMT (resembles Luminal subtype in humans). The suppression of H3K27 trimethylation was not significant with GSK126, suggesting that the basal subtype may be more sensitive to the drug (**Fig. 6F**). We have also used additional basal human cell lines (HCC1806, SUM-159, MDA-468). GSK126 efficiently inhibited H3K27 trimethylation (**Fig. 6**).

Impact of EZH2 inhibition on various TNBC models: *1)* HCC1806 model: GSK126 effectively blocked EZH2-mediated H3K27me3 in a dose dependent manner and impaired invasion across a matrix coated transwell membrane in the HCC1806 TNBC models (**Fig. 7A-B**). Consistent with the MDA-MB-LM2 model, GSK126 did not impact primary tumors (**Fig. 7C**). However, in this model, we found out that the metastatic

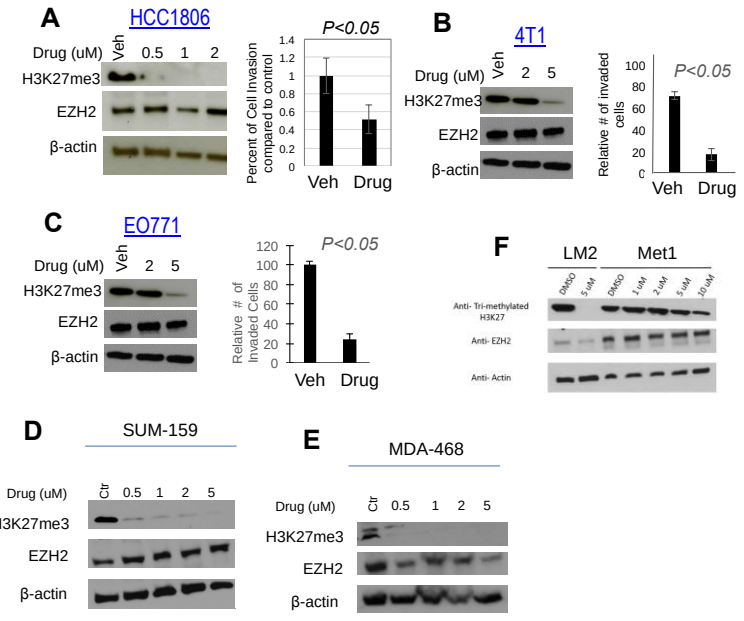

Fig. 6 (A-E) TNBC lines respond to EZH2 inhibition as determined by reduced H3K27me3 levels and invasion through a matrix coated transwell, **(F)** Met1 responds poorly to EZH2 inhibition.

penetrance was extremely low (**Fig. 7D**), as there was not BLI in the lungs (data not shown), and evaluation of lungs by IHC did not reveal visible metastatic lesions. Therefore, the HCC1806 model, does not allow us to determine the impact of EZH2 inhibition on metastasis.

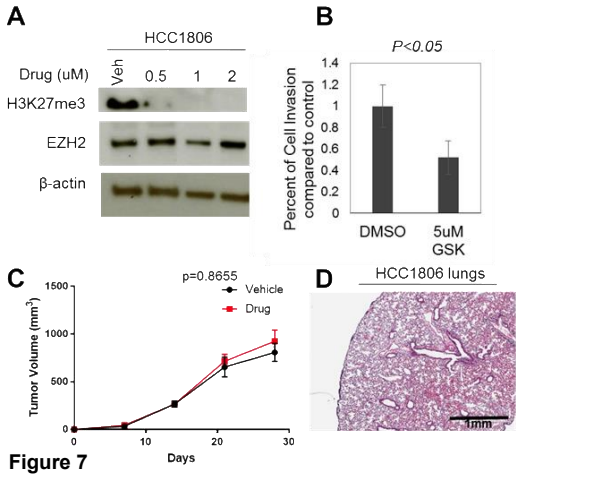

Figure 7

EZH2 HMT inhibition impairs invasiveness of HCC1806 breast cancer model without impacting primary tumor growth.

(A) GSK126 suppresses EZH2-mediated H3K27me3 in HCC1806 cells.
(B) GSK126 impairs invasion through a matrix coated membrane.
(C) GSK126 has no impact on primary tumor size in vivo.
(D) No lung macrometastases in the HCC1806 breast cancer model

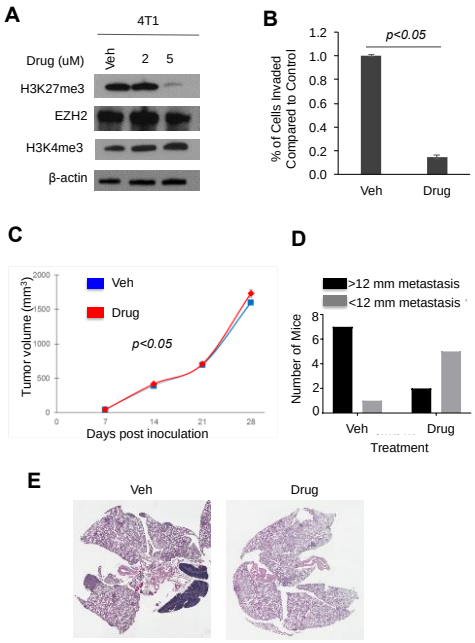

Figure 8. EZH2 HMT inhibition impairs breast cancer metastasis to the lung in the 4T1 breast cancer model. **(A)** EZH2 inhibition suppresses H3K27me3 in 4T1 cells. **(B)** EZH2 inhibition impairs invasion through a matrix coated membrane. **(C)** EZH2 inhibition impairs invasion through a matrix coated membrane. **(D)** EZH2 inhibition impairs lung mets. Shown are # of mice with >12 mm lesions or <12 mm lesions. **(E)** H and E of a representative lung from D.

<u>2) 4T1 model:</u> As another TNBC model, we tested the impact of EZH2 inhibition on the 4T1 tumor growth. The 4T1mouse mammary tumor model closely mimics human breast cancer in its anatomical site, immunogenicity, growth characteristics, and metastatic potential. EZH2-mediated suppression of H3K27me3 in a dose dependent manner and impaired invasion across a matrix coated transwell membrane (**Fig. 8A-B**). Next, 1×10^6 viable 4T1 breast cancer cells stably expressing luciferase and RFP reporter transgenes were injected into the mammary gland of immunocompetent BALB/c mice. Primary tumors were allowed to grow and GSK126 was administered intraperitoneally (IP) at 150 mg/kg twice per week (n=10 mice per group) for 2 weeks. Primary tumors were surgically resected at X weeks and animals monitored for metastasis. Consistent with the MDA-LM2 model, GSK126 also did not impact primary tumor growth (**Fig. 8C**) but significantly impaired lung metastasis (**Fig. 8D-E**).

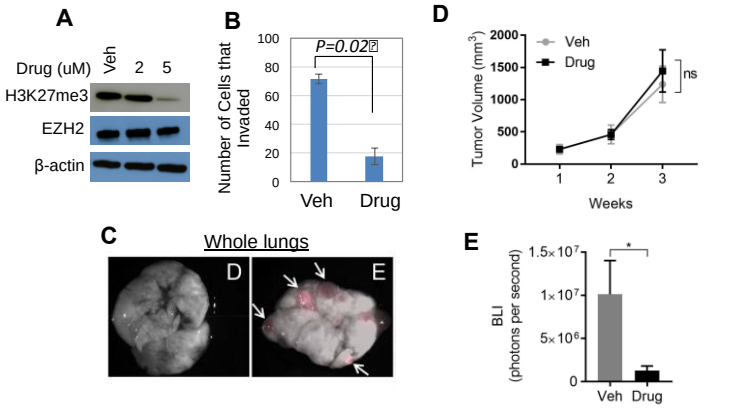

Fig. 9 EZH2 inhibition does not regress primary tumors but impairs metastasis in EO771.LMB model. **(A)** Western blots showing H3K27me3 and EZH2 in EO771 cells treated with Veh or drug. **(B)** Invasion assays through a matrigel coated transwell. **(C)** E0771 implanted orthotopically in the mammary glands of immunocompetent C57BL/6 mice metastasize to lungs (Panel C is *from Jonstone et al. Dis Model Mech 2015).* **(D)** GSK126 (150mg/kg, twice per week) does not impact primary orthotopic tumors. **(E)** BLI counts of metastatic lungs. * denotes significance, p<0.05

<u>3) EO771 immunocompetent model:</u> Consistent with HCC1806 and 4T1 models, the immunocompetent metastatic TNBC mouse model, E0771.LMB[31], showed that GSK126 suppressed H3K27me3 and invasion (**Fig. 9A-B**). However, in vivo (**Fig. 9C**), GSK126 did not impact primary tumor growth, however, it significantly impaired metastasis (**Fig. 9D-E**)

Together, data from multiple independent TNBC models (MDA-LM2, HCC1806, 4T1 and EO771) suggests that EZH2 inhibition may target the metastatic cells in primary breast tumors. Demonstration that EZH2 blockade impacts metastasis in multiple metastatic models establishes the use of EZH2 inhibition as anti-metastatic therapy.

To determine the mechanisms by which EZH2 inhibition caused metastasis suppression, we performed performed ChIP for H3K27me3 coupled with qRT-PCR to characterize the effect of GSK126 on repressive histone mark H3K27me3. This enabled us to identify additional EZH2 downstream targets. Among several candidates (**Fig. 10**), we observed increased occupancy of

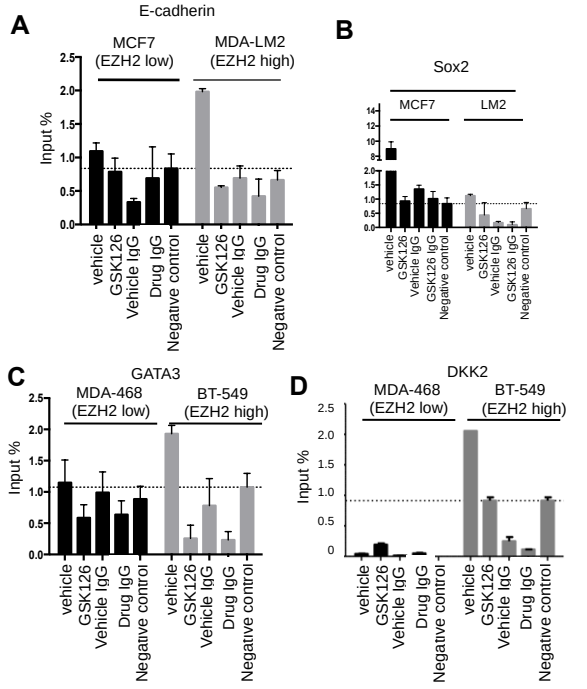

Fig. 10. H3K27me3 ChIP and q-PCR of selected promoters. ChIP-qPCR showing % input of H3K27me3 at E-cadherin (**A**), Sox-2 **(B**) GATA3 (**C**) and DKK2 (**D**) promoter in metastatic breast cancer cells indicated. IgG was used as control. No enrichment observed in EZH2 low non-metastatic cells.

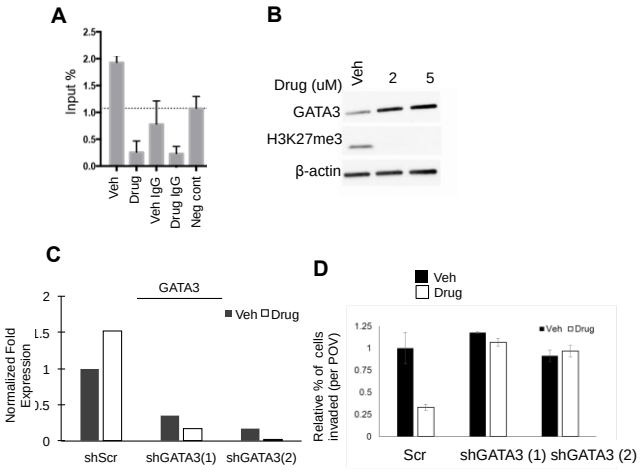

Fig. 11. EZH2 silences GATA3 expression. (A) ChIP-qPCR showing H3K27me3 on GATA3 promoter in vehicle and drug treated cells. **(B)** EZH2-mediated Gata3 silencing (two shRNAs) is reversed with EZH2 inhibition. **(C)** EZH2 inhibition loss of invasion is Gata3 dependent.

H3K27me3 on the GATA3 promoter in vehicle treated cells compared to treatment with GSK126 drug (**Fig. 11A**). Consistent with this finding, EZH2 inhibition restored GATA3 expression (**Fig. 11B**). Indeed, loss of invasion phenotype resulting from EZH2 inhibition was abrogated in GATA3 knockdown compared to scrambled shRNA (SCR) (**Fig. 10C**), implicating the EZH2-GATA3 axis

Subtask 2: Measure levels of miR-708, Nnat, cell migration and invasion.

Subtask 3: Determine if PRC2 blockade impacts metastasis through miR-708. (Perform miR-708 ablation either using sponge or CRISPR method in MDA cells) and determine if pharmacological blockade of EZH2 is incapable of metastasis suppression.

We have demonstrated that 1) EZH2 blockade impairs metastasis, 2) miR-708 is suppressed in metastasis and miR-708 overexpression impairs metastasis, and 3) EZH2 epigenetically suppresses miR-708 expression.

To establish, whether EZH2-miR-708 axis is the dominant pathway in breast cancer metastasis, we used a miR-708 antigomir that specifically inhibits mir-708. Notably, GSK126 mediated upregulation

of miR-708 was blocked by miR-708 antigomir compared to a scrambled antigomir control (**Fig. 12A**). The miR-708 antigomir did not impact GSK-126 mediated H3K27 trimethylation levels (**Fig. 12B**). Importantly, miR-708 antigomir overcame the invasive suppressive ability of GSK126 on LM2 cells **(Fig. 12C**).

To establish a stable suppression of miR-708 in cancer cells, we began to generate miR-708 knock out cells using the CRISPR/Cas9 approach in order to confirm our above results in vivo (**Fig. 13**). Clone 3 was identified for further characterization. Unfortunately, further analysis unraveled that Clone 3 did not have a defined CRISPR generated mutation in miR-708 and we have decided not to proceed with this clone. We have designed new CRISPR guides to completely delete the miR-708 locus. This analysis will be a part of future investigations.

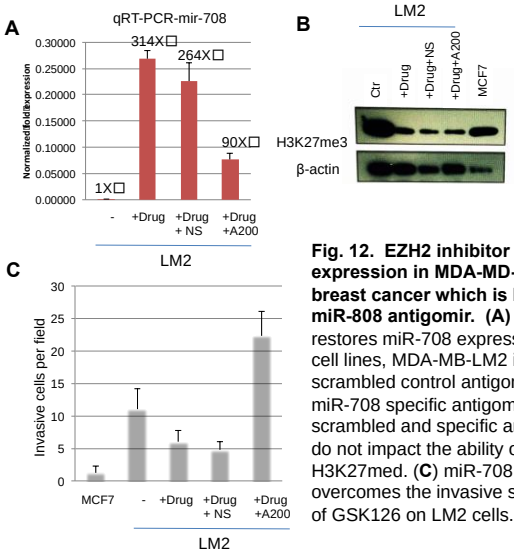

Fig. 12. EZH2 inhibitor restores miR-708 expression in MDA-MD-LM2 metastatic breast cancer which is blocked by miR-808 antigomir. (A) 5 μM GSK126 restores miR-708 expression in metastatic cell lines, MDA-MB-LM2 in the presence of scrambled control antigomir but not with miR-708 specific antigomir. (**B**) The scrambled and specific antigomir sequnces do not impact the ability of GSK126 to block H3K27med. (**C**) miR-708 antigomir overcomes the invasive suppressive ability of GSK126 on LM2 cells.

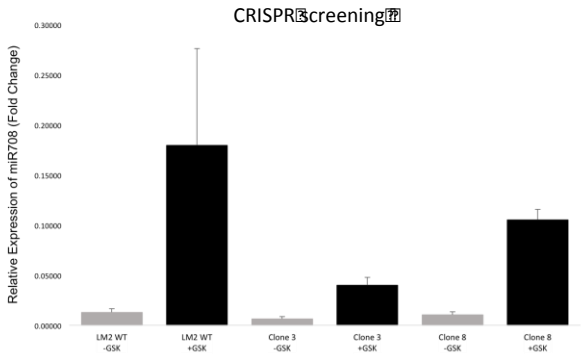

Fig. 13. miR-708 knockout using CRISPR/Cas system. 5 μM GSK126 restores miR-708 expression in control metastatic cell line, MDA-MB-LM2, but not in =Clone 3.

Aim 2

Aim 2 Major Goal 1: Assess the therapeutic potential of miR-708 against metastatic TNBC breast cancer

Subtask 1: MDS for delivery of miR-708 to prevent metastases derived from orthotopic tumors.

We focused on the delivery of miR-708 in vivo. However, we had some initial setbacks in the delivery of miR-708 with the liposome method. So as indicated in the alternative aims, we have explored the use gold nanoparticles as delivery vehicles for miR-708. In this context, we have had success in the optimization of these particles. miR-708 coated nanoparticles (**Fig. 14A**), incubated with cells in vitro, showed suppression of miR-708 target NNAT, compared to nanoparticles carrying scrambled miRNAs (**Fig. 14B**). These findings suggest that gold nanoparticles can deliver the miR-708 payload in cells.

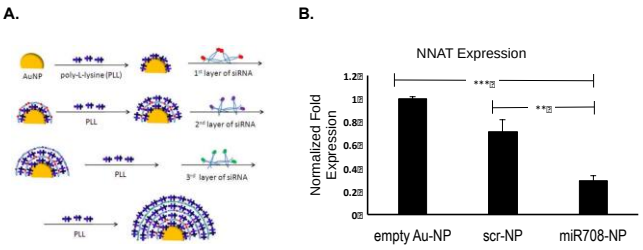

Fig. 14. Nanoparticle delivery of synthetic miR-708 targets NNAT. A) Preparation of multi-layered siRNA coated gold nanoparticles using alternative positively charged PLL and negatively charged siRNA layers (Ref. Lee et al., *Small*, 2011) **B) C**ells expressing miR-708 target NNA were incubated withnanoparticles: either empty gold (empty Au-NP), or scrambled (scr-NP), or microRNA-708 (miR708-NP) in triplicates. After 48 hours, RNA was extracted from the cells and NNAT expression was measured via qPCR. Statistics were performed by one-way ANOVA, followed by Tukey's test. P-value = 0.0009

In the no-cost extension period, we explored nanoparticle delivery in vivo. Mice bearing LM2 tumors in the mammary fat pad, were administered gold nanoparticles layered with siRNA targeting luciferase gene and labeled with Cy5.5. This experimental design (**Fig. 15A**) would allow us to track homing of the particles in vivo to the tumor site and also assess if the siRNA can be delivered to the primary tumors, as determined by suppression of luciferase gene. Indeed, nanoparticles were observed at the primary tumor site as determined by Cy5.5 fluorescence (**Fig. 15B**). This accumulation of particles in the primary tumors as associated with suppression of luciferase signal (**Fig. 15C**). Together these findings, have encouraged us to package miR-708 in these particles to determine impact on metastasis suppression.

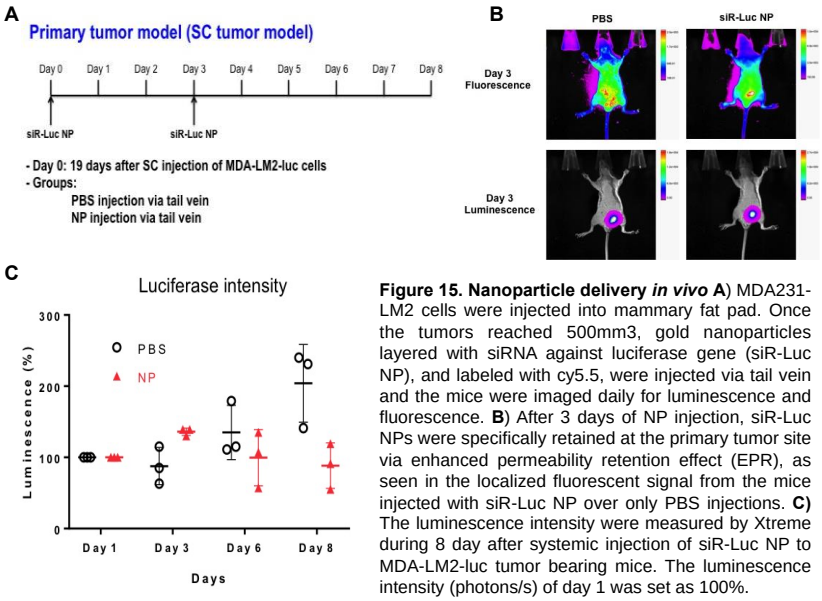

Figure 15. Nanoparticle delivery *in vivo* A) MDA231-LM2 cells were injected into mammary fat pad. Once the tumors reached 500mm3, gold nanoparticles layered with siRNA against luciferase gene (siR-Luc NP), and labeled with cy5.5, were injected via tail vein and the mice were imaged daily for luminescence and fluorescence. **B**) After 3 days of NP injection, siR-Luc NPs were specifically retained at the primary tumor site via enhanced permeability retention effect (EPR), as seen in the localized fluorescent signal from the mice injected with siR-Luc NP over only PBS injections. **C)** The luminescence intensity were measured by Xtreme during 8 day after systemic injection of siR-Luc NP to MDA-LM2-luc tumor bearing mice. The luminescence intensity (photons/s) of day 1 was set as 100%.

In order to explore the efficacy of nanoparticle co-localization with lung metastases *in vivo*, we utilized an experimental metastasis model of LM2 cells, injected via tail vein in 8-week old mice **(Fig. 16A)**. Once the lung metastases were established, we injected these mice with nanoparticles layered with siRNA for luciferase reporter (siR-luc), and labeled with cy5.5 fluorescence marker. BLIs and fluorescence for the mice were measured **(Fig. 16B)**. After three days of the first injection, we found that the co-localization of nanoparticles in the tumor-bearing lungs was higher than muscle or spleen **(Fig. 16C)**.

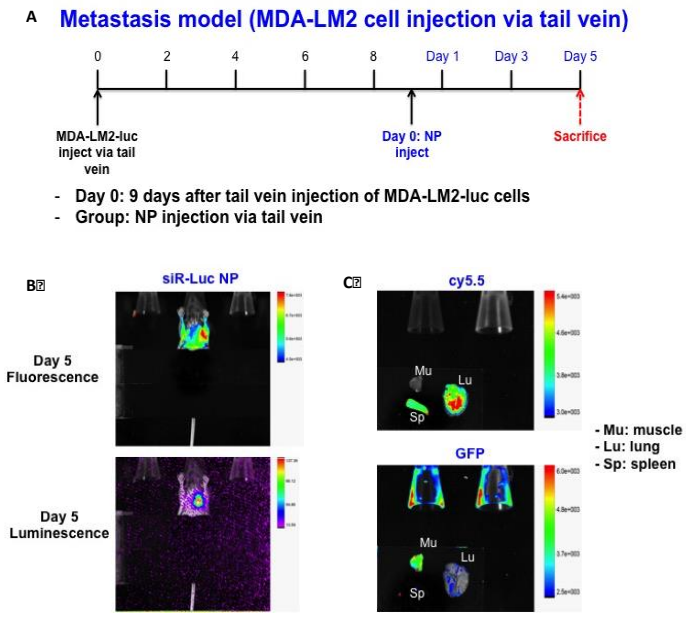

Fig. 16 A) **Experimental Metastasis Model.** 500k MDA231-LM2 cells were injected in 8-week old female SCID mice via tail vein. Once the luminescence signal was stabilized in these mice, cy5.5-labeled siR-Luc NPs were injected via tail vein and the mice were imaged every day for fluorescence and luminescence. B and C) **Localization of NPs in lungs.** At day 5, localized fluorescence signal was seen in tumor-bearing lungs. A comparison of fluorescence and luminescence signals from lungs as seen in comparison with muscle and spleen. Liver had the highest fluorescence signal from NPs, due to clearance of these particles via liver (data not shown).

Next, we explored the consequences of nanoparticle-mediated mir-708 delivery on primary tumor progression and metastasis *in vivo*. For this, we utilized LM2 primary TNBC model. At week 4, mice were injected with gold nanoparticles layered with either with scr (scr-NP) or miR708 (miR708-NP) oligos. These nanoparticles were labeled with cy5.5, to track their localization, and were injected via tail vein, twice a week for two weeks. The mice were imaged for luminescence and fluorescence. Nanoparticles (both scr-NP and miR708-NP) co-localized well at the primary tumor site

(Fig. 17A). As observed before (Ryu 2013), miR-708 had no effect on the primary tumor size **(Fig. 17C)**. Primary tumors were resected once they reached 1 cm^3, mice were allowed to recover, and then nanoparticle injections were continued for two weeks. During this time, lung metastases were analyzed using BLI. In the scr-NP group, mice developed significantly more lung metastases as compared to miR708-NP group **(Fig. 17D)**, and the localization of nanoparticles in the lungs of mice with more metastases was seen **(Fig. 17B)**. To ensure effective activity of miR708, bulk primary tumors from the two cohorts (scr-NP and miR708-NP) were analyzed for the levels of miR708 and its downstream target NNAT by qPCR. We found that the group treated with miR708-NP had higher levels of the microRNA **(Fig. 17E)** and correspondingly lower levels of NNAT **(Fig. 17F)** than scr-NP group.

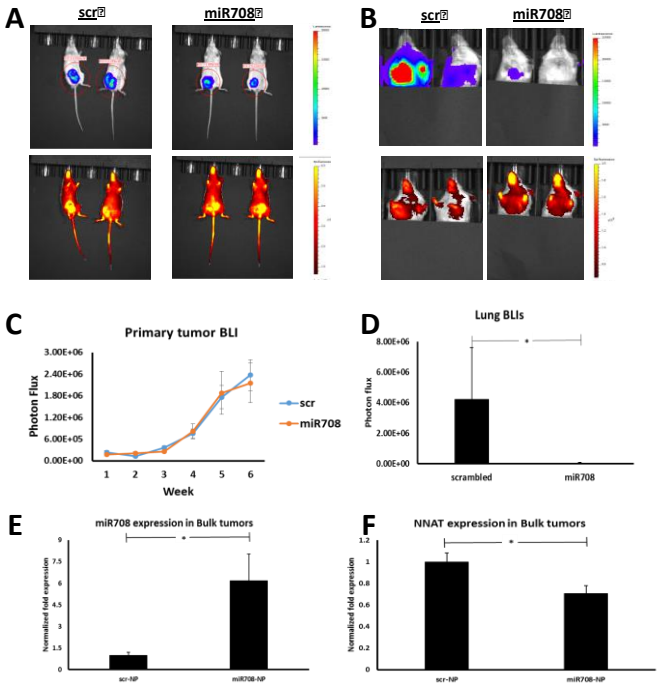

Fig.. 17. (A,C) Nanoparticle (SCR and mir708) co-localized at the primary tumor site, with no change in primary tumor volumes in either group. **(B, D) Lung metastases.** Nanoparticle accumulation in the lungs of mice with larger metastases can be seen. There was a significant reduction in the total photon flux from the lungs of mice treated with miR708-NP. **(E,F) *In vivo* gene-silencing effect**. Primary tumors from the mice treated with miR708-NP had higher miR708 expression, and as expected, correspondingly lower NNAT expression than mice treated with scr-NP. *p<0.05

Subtask 2: MDS for delivery of miR-708 to treat heterotransplanted patient breast cancer.

After having established the impact of EZH2 HMT inhibition on breast cancer metastasis in cell line-derived breast cancer models, we were interested in assessing the therapeutic benefit of EZH2 inhibitor, using a "co-clinical animal trial" design that utilizes PDX models of TNBC. Notably, these PDX models are serially propagated in mice and have maintained their triple negative marker status, molecular profiles and histological features of original human specimens. Notably, 9/21 TNBC models metastasized to the lung in concordance with patient nodal/metastatic status (Liu et al., 2010; Zhang et al., 2013). Importantly, we have determined that PDX tumors with metastatic potential in both patients and mice are associated with elevated EZH2 levels (8/8), and tumors with low metastatic potential are associated with low EZH2 (4/6) (**Fig. 18**)

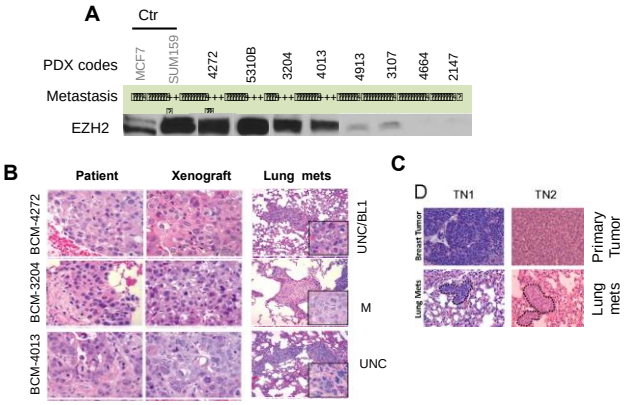

Fig. 18. Breast cancer TNBC PDX models. (A) Western blots showing EZH2 levels (normalized to actin) in a collection of TNBC PDX models. Controls are MCF7 and SUM159 cells **(B)** Histology of representative PDX models that metastasize to lungs, * indicates models that will be used in this study (obtained from *Dr. Chang*; *Zhang et al. Cancer Research 2013*. **(C)** TN1 and TN2 TNBC models showing primary tumors and lung mets (obtained from *Dr. Clarke, Liu et al. PNAS 2010*)

Given that, we had optimized the nanoparticle delivery of miR-708 in the no-cost extension year, it became challenging to perform similar experiments in PDX models. Therefore, to establish proof of concept that the TNBC PDX models respond to EZH2 inhibition we performed GSK126 treatment.

We generated orthotopic in the mammary glands of NOD-*scid IL2rγ*^{null} (NSG) mice (n=10/group) from two PDX lines representing Basal like 2 and Mesenchymal like subtyps. Primary tumors were treated with GSK126 (150mg/kg, twice a week), and surgically resected (1cm^3, about 6-8 weeks), and metastasis was monitored. Primary tumors (-/+ drug) were dissociated into single cells and subjected to flow cytometry for TICs (SOX2) and mammosphere

formation. Consistent with the cell line model, EZH2 inhibition did not impact primary tumor growth in both PDX models (**Fig. 19A**).Notably, EZH2 inhibition reduced lung metastasis (red arrows, **Fig. 19B**). These preliminary results provide an ideal platform to test whether EZH2 inhibition or delivery of miR-708 would impair metastasis in various TNBC molecular subsets.

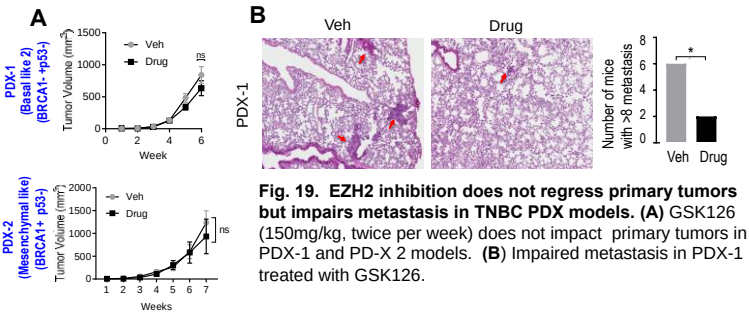

Fig. 19. EZH2 inhibition does not regress primary tumors but impairs metastasis in TNBC PDX models. (A) GSK126 (150mg/kg, twice per week) does not impact primary tumors in PDX-1 and PD-X 2 models. **(B**) Impaired metastasis in PDX-1 treated with GSK126.

What opportunities for training and professional development has the project provided?
Opportunities for training and professional development on the project include the mentorship of graduate students and post-doctoral associates to help advance their careers.

How were the results disseminated to communities of interest?
Throughout the period of the award, Dr. Mittal has attended annual cancer-associated conferences and several national and international invited seminars to communicate the existence of this project and the intended goals.

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

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?
Our study has the potential to establish EZH2 blockade or delivery of miR-708 as an attractive therapeutic approach for treatment of metastatic breast cancer including TNBC, for which FDA approved targeted therapies are lacking.

What was the impact on other disciplines?
This is the first study to demonstrate the impact of EZH2 inhibitor (GSK126) or miR-708 in breast cancer metastasis. This is likely to attract many investigators across disciplines in breast cancer research and result in rapid advancements towards finding a potential therapy for TNBC.

What was the impact on technology transfer?
miR-708 technology is covered in both mechanism of action and composition of matter (PCT/US2013/066376). Based on more recent findings, we will determine if EZH2-miR-708 axis has a potential for IP filings.

What was the impact on society beyond science and technology?
Nothing to report

5. CHANGES/PROBLEMS:

Nothing to report

6. PRODUCTS:

Two manuscripts are under preparation/submission. One described the nanoparticle delivery of miR-708 to target TNBC metastasis in preclinical models. The other describes studies related to EZH2 inhibition in TNBC metastasis.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Vivek Mittal (PD) – No change*
Name:	*Linda Vahdat (Co-Investigator) – No change*
Name:	*Jenny Chang (Co-Investigator) – No change*
Name:	*Melissa Landis (Post-Doc) – No change*

Name:	*Divya Ramchandani (Post-Doc) – During Extension Year*
Project Role:	*Post-Doc*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*3.6 months over a 12 year period*
Contribution to Project:	*Dr. Ramchandani has performed all mir708 manipulations and spearheaded the experiments and all troubleshooting*
Funding Support:	

Name:	*Sharrell Lee (Technician) – All Years*
Project Role:	*Technician*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*5.65 months over a 4 year period*
Contribution to Project:	*Ms. Lee has assisted in vivo work*
Funding Support:	

Name:	*Seongho Ryu (Post-Doc) – In Year 1*
Project Role:	*Post-Doc*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1.8 over a 12 month period*
Contribution to Project:	*Dr. Ryu has performed all mir708 manipulations and spearheaded the experiments and all troubleshooting*
Funding Support:	

Name:	*Lauren Havel (Post-Doc) – in Year 2 & 3*

Project Role:	*Post-Doc*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*7.2 months over a 24 month period*
Contribution to Project:	*Dr. Havel has performed all mir708 manipulations and spearheaded the experiments and all troubleshooting*
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?

1) Dr. Mittal received a one-year research award from the Weill Cornell Medical College's Meyer Cancer Center entitled, "Circulating tumor DNA for early detection and management of non-small cell lung cancer." The dates are from 12/01/2016 – 11/30/2018. He is spending 0.83% effort (0.1 calendar months).
2) Dr. Mittal received a one-year research pilot sub-project 5U54CA210184 from Cornell University PSOC in partnership with the Meyer Cancer Center entitled, "Mechanisms of copper depletion in TNBC metastasis- from bedside to the bench" with Dr. Claudia Fischbach at Cornell University (Ithaca, NY) and Dr. Noah Dephoure at Weill Cornell (New York, NY). Dates are from 09/15/2017 – 09/14/2018. He is spending 10% effort (1.2 calendar months).

What other organizations were involved as partners?

Organization Name: The Methodist Hospital Research Institute (TMHRI)
Location of Organization: Houston, TX
Partner's contribution to the project: Collaboration – TMHRI is a subawardee on the award.

8. **SPECIAL REPORTING REQUIREMENTS**

Nothing to report

9. **APPENDICES:**

Nothing to report