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14. ABSTRACT A substantial proportion of breast cancer patients develop metastases despite surgeries and adjuvant therapies. Metastasis is incurable and responsible for over 90% of breast cancer-related death. Thus, the prevention of metastasis is an imperative clinical need. We seek to understand how microscopic metastases in distant organs (e.g., bone), before becoming overt malignancies, survive and progress by interacting with specific normal cells in that organ. The rationale is that such interaction may confer resistance to current adjuvant therapies and may also render the cancer cells vulnerable to novel treatments. To date, very few pre-clinical models of micrometastases exist. We have filled this gap by developing a series of techniques that allow us to monitor and quantitate the progression of micrometastases. In this application, we will further establish the authenticity of these models in reflecting biological properties of micrometastases. We will also use them to identify therapies that may eliminate metastatic seeds, especially in the bone. We will examine all breast cancer subtypes with an emphasis on estrogen receptor-positive breast cancer and investigate how the bone environment influences cancer cells' response to endocrine therapies. Specifically the three goals are: 1) To assess the differential responses of bone micrometastases to adjuvant therapies as compared to their parental tumors in the mammary gland, and dissect if and how such differences are attributable to the interaction with their adjacent normal cells.; 2) to further establish an experimental platform called "Bone-in-culture array" (BICA) that can mimic bone micrometastases and allow rapid testing of drug efficacies; and 3) to perform drug screening/discoveries to identify compounds that can be combined with current standard-of-care and eradicate bone micrometastases. The fulfillment of these goals will provide novel strategies that may significantly reduce bone or possibly other metastases in breast cancer. Moreover, the same techniques can be easily applied to other cancer, including lung cancer and some pediatric sarcomas.					
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1. Introduction

In this project we aim to overcome the challenge of eliminating microscopic metastases of breast cancer, so that distant recurrences and related deaths can be significantly reduced in the foreseeable future. We will focus on bone micrometastases (BMM), which are precursors of overt bone metastases and possibly other metastases. In particular, we will delineate how breast cancer cells, when isolated in small quantity in a foreign milieu, react to therapies differently compared to the original primary tumor. We have designed and will continue to optimize various pre-clinical models to investigate the microenvironmental effects on BMM. These models will enable medium-throughput drug discovery/repositioning to expedite the elimination of breast cancer cells in the context of bone. The methodology may also be applied to metastases in other sites.

In the clinic, primary breast tumors are usually surgically removed soon after diagnosis, often leaving patients “tumor-free”. However, 20-40% of breast cancer survivors will eventually suffer metastasis to distant organs, sometimes years after surgeries. Thus, the life-threatening enemy is typically not the bulk of primary tumors, but the dispersed metastatic seeds left behind, which have already disseminated to distant organs, may be temporarily dormant, and may resume aggressive outgrowth under certain yet-to-be-identified conditions. Current adjuvant therapies intend to eliminate these cells. However, the therapeutic decisions and strategies are usually based upon pathological features of primary tumors. Micrometastases are likely to differ from their parental primary tumors due to Darwinian selection and/or adaptation in a different milieu. In either case, the microenvironment in distant organs plays a critical role in driving the selection and/or in shaping the adaptive reaction of cancer cells. It is our vision that a critical barrier in curing breast cancer is the lack of knowledge about micrometastases and their microenvironment niches. Specifically, the key questions are the nature of the supporting pathways uniquely induced by cancer-niche interaction, and the mechanisms responsible for differential therapeutic responses as compared to parental primary tumors. To overcome this barrier, I propose to establish a series of pre-clinical models that recapitulate the cellular nature of micrometastases, mimic their habitat and allow expedited testing of their drug responses.

Three specific aims will be pursued. 1. To assess the differential responses of BMM to adjuvant therapies as compared to their parental tumors in the mammary gland, and dissect if and how such differences are attributable to the interaction with the microenvironment niche. 2. To establish the bone-in-culture array (BICA) platform, which aims to faithfully recapitulate the molecular profile, cell-biological behaviors, microenvironment niche, and therapeutic responses of BMM in vivo, and is amenable to medium-to-high throughput drug discovery/screening. 3. To identify and mechanistically investigate therapies against BMM by analyzing the omics data obtained from previous goals, and by screening pre-established libraries of FDA-approved drugs or small molecule inhibitors (SMIs).

2. Keywords:

Metastasis, microenvironment, drug discovery, therapeutic resistance, micrometastases, endocrine resistance

3. Accomplishment

Major Task 1 : Differential drug responses of bone micrometastases (BMM) as compared to the parental orthotopic tumors

Subtask 1: Tumor burden measurement (Month 1-24). We expect to use five PDXs (2 ER+, 1 Her2+ and 2 triple negative) and five cell lines (the same subtype distribution). The total # of models will be 10. Each model will need 55 mice. PDXs will be transplanted into SCID/Beige mice and cell lines will be transplanted into Athymic nu/nu mice. These mice will be divided

into treated and untreated. Treatments: tamoxifen, fulvestrant, ovariectomy, and lapatinib. Measurement: Weekly bioluminescence imaging and tumor volume measurement. Some mice will be euthanized at intermediate time points for Subtasks 2 and 3 below.

We have successfully collected and validated all experimental models. In particular, the 2 ER+ PDX models have been validated to express ER and responsive to estrogen treatment. The responses to endocrine therapies have been tested for some of the cell lines as orthotopic tumors including MCF-7, MDA-MB-361 and MDA-MB-231. These data will be provided together with other models in the next report.

Subtask 2: Immunofluorescence staining to quantitate proliferation (e.g., Ki67+), survival (e.g., CC3) and self renewal (e.g., retention of H2B-GFP) (Month 1-18).

Again, this has been done in cell lines including MCF-7. We will soon begin this work on PDX models. The results will be collected and presented in the next report period.

Subtask 3: Gene expression profiling on orthotopic tumors and BMM (subjected to various treatments) to deduce differential responsive genes/pathways (Month 12-24).

To begin soon.

Major Task 2: Test if the abolishment of cancer-niche interaction in conditional N-cadherin KO mice reverses the therapeutic responses of BMM.

Subtask 1: Mouse breeding to generate animals with various genetic background (including immunodeficiency). We will breed TetO-Osx-cre-GFP with *Cdh2^{fl/fl}* mice both purchased from Jackson Laboratories (Stock No: 006361 and 007611, respectively) to generate offsprings with both genetic alterations. The mice will also be crossed with *Rag1^{-/-}* mice (Stock No: 002216) to generate immunodeficiency for human cancer cell transplantation. (Month 1-24).

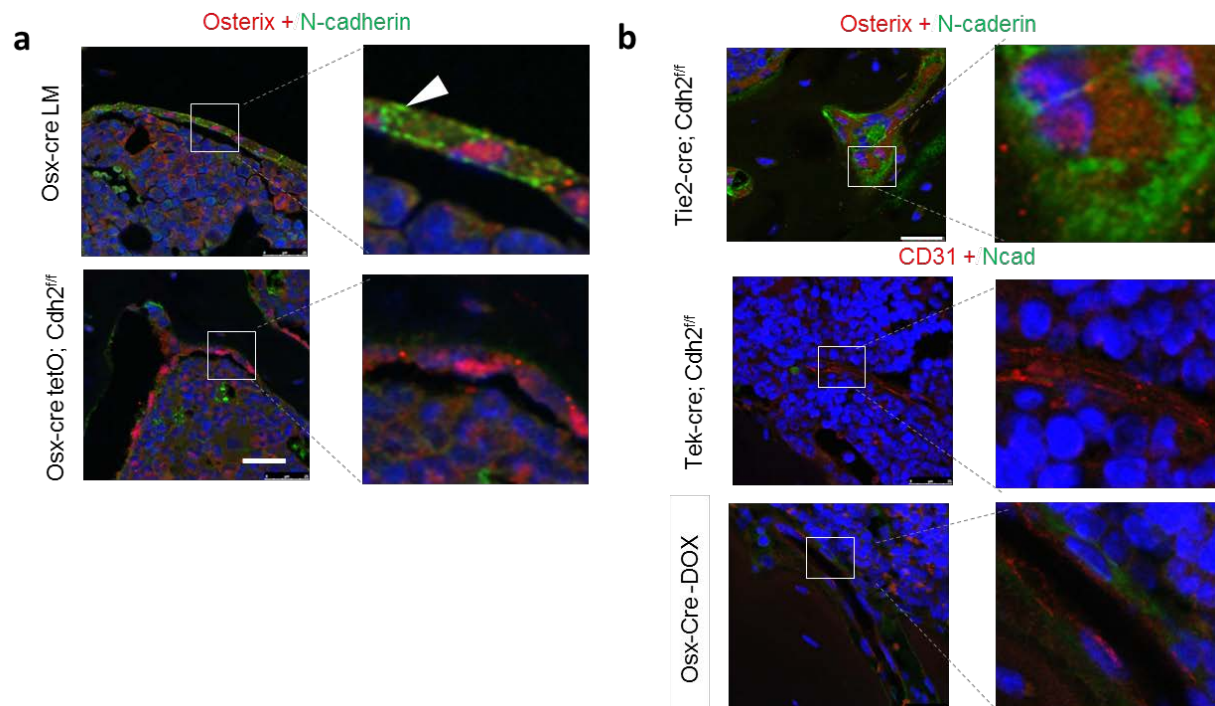


Figure 1: Validation of mouse models with BMM niche abolished by genetic knockout. (a) Representative pictures demonstrating the conditional knockout of N-cadherin in Osterix+ cells in the bone of *Osx-cre tetO; cdh2^{fl/fl}* mice or the litter mate (LM) control with only *osterix-cre*. Red: Osterix. Green: N-cadherin. Scale bar: 50 µm. (b) Representative fluorescence staining shows conditional knockout of N-cadherin in endothelial cells. Top row: red- osterix+ cells, green- N-cadherin. Middle and bottom rows: red- CD31+ cells, green- N-cadherin. Scale bars: 25 µm.

This work has been ongoing and progressing smoothly. We have successfully obtained mice with the desired genotype. We have also validated the conditional and inducible knockout of *Cdh2* gene (Figure 1). We are now in the process crossing it with *Rag1*^{-/-} mice for human cancer cells. These data will be reported in the grant period.

Subtask 2: Repeat experiments in Major Task 1 in the conditional N-cadherin KO models, and Test if the abolishment of cancer-niche interaction in conditional N-cadherin KO mice reverses the therapeutic responses of BMM. TetO-Osx-cre-GFP; *Cdh2*^{f/f} mice and TetO-Osx-cre-GFP; *Cdh2*^{f/f}; *Rag1*^{-/-} mice and their littermate female mice lacking Osx-Cre will be subjected to experiments as in Task #1. About 700 mice will be bred at this stage. Except that a few male mice carrying the wanted phenotype will be kept for strain maintaining, most male mice (estimated to be 340) will be euthanized right after genotyping. (Month 24-36)

To begin by Month 24.

Major Task 3: To establish and validate BICA

Subtask 1 : Characterize the cell-biological features of cancer cells in BICA (e.g., proliferation, self-renewal, and survival). We expect to use five PDXs (2 ER+, 1 Her2+ and 2 triple negative) and five cell lines (the same subtype distribution). The total # of models will be 10. Each model will need 20 mice. PDXs will be transplanted into SCID/Beige mice and cell lines will be transplanted into Athymic nu/nu mice. (Month 1-18)

As mentioned in Major Task 1, all models have been obtained and validated in the past grant period. We have fulfilled BICA development using MCF-7 and MDA-MB-368 cell lines, and will soon move on to other models.

Subtask 2: To characterize the microenvironment niche in BICA using different subtypes of cancer models. This will be achieved by immunohistochemical and immunofluorescence staining of the following markers: ALP, Col-I, CTSK, Osterix, Runx2, CD31, NG2, and SOX9. The same numbers and PDXs and cell lines will be used as specified in Subtask 1 above. (Month 1-24).

Similar to above tasks, this work has been mostly fulfilled using MCF-7 cells as the first model, and will soon be extended to other models.

Subtask 3: To perform RNA-seq of cancer cells in BICA, and compared the profiles to cancer cells in intact bones and in mammary glands. For this task we will use 2 PDX (1 ER+ and 1 Her2+) and 2 cell lines (1ER+ and 1 Her2+). Each will be injected into 25 animals (5 for orthotopic tumors, 10 for intact bone metastases, and 10 for BICA). (Month 18-36)

To begin by Month 18.

Subtask 4: To determine the therapeutic responses of cancer cells in BICA as compared to those of BMM in vivo and cancer cells in culture. We expect to use five PDXs (2 ER+, 1 Her2+ and 2 triple negative) and five cell lines (the same subtype distribution). The total # of models will be 10. Each model will need 50 mice. PDXs will be transplanted into SCID/Beige mice and cell lines will be transplanted into Athymic nu/nu mice. (Month 30-48)

To begin by Month 30.

Major Task 4: Analyze the RNA-seq data obtained from Specific Aim 1 to identify and validate candidate pathway/genes that can be targeted to eliminate BMMs

Subtask 1: Bioinformatics analyses to identify candidate pathways/genes. (Month 1-36)

We have begun to collaborate with Dr. Quincy Mo (Co-I of the project) to perform proof-of-principle analyses on mock datasets. We have also identified important software packages that

would be later needed for proposed analyses. The actual analyses will begin soon after the generation of the first batch of RNA-seq data (Major Task 3, Subtask 3).

Subtask 2: Select candidates for functional validation in vivo and in BICA. (Month 36-60)

To begin by Month 36.

Major Task 5: Screening of small SMI libraries to identify FDA-approved drugs or new compounds that can eliminate BMM.

Subtask 1: Screening using BICA. We will use one ER+ PDX and one ER+ cell lines. Each model will be applied to 100 mice. This will generate approximately 5000 bone fragments, and can be used for screening of small drug libraries described in the proposal. Four libraries and BICA-screening will be performed. (Month 24-36)

To begin by Month 24.

Subtask 2: Identify and validate the efficacies of top candidates on BMM. We will use the same models as subtask 1. (Month 30-36)

To begin by Month 30.

Subtask 3: Optimize and modify the compounds to achieve higher efficiency. We will use the same models as subtask 1. (Month 36-48)

To begin by Month 36.

Subtask 4: In-depth mechanistic studies of the validated compounds. We will perform RNAseq on BMM in vivo to delineate pathways affected by the compounds. We will then identify key genes that may mediate the compounds' effects. Genetic depletion will then be performed to perturb these genes. The models are the same as subtask 1. (Month 36-60)

To begin by Month 36.

4. Impact

The proposed research is innovative and distinctive in the field of breast cancer research for the following reasons. First, to date few experimental models can be used to test the therapeutic responses of BMM. The vast majority of pre-clinical research has been done using orthotopic tumor models, despite the fact that micrometastases are the major targets of adjuvant therapies. We have established unique in vivo and ex vivo models to fill this gap. We will use these models to elucidate how different subtypes of breast cancer respond to their respective adjuvant therapies as microscopic lesions embedded in the bone, a significant step toward full recapitulation of clinical scenarios. Second, BICA combines the complexity of bone microenvironment and the scalability of in vitro culturing. Compared to previous "tissue-in-culture" approaches, bone-in-culture represents a better mimicry of the counterpart organ because BMM are tightly integrated into the osteogenic niche and are difficult to be dissociated from the bone tissue. As a result, the cancer-niche crosstalk is preserved after tissue fragmentation. Thus, BICA provides distinctive opportunities to rapidly assay hundreds of compounds and reveal novel treatments of BMM. Third, the proposed research assembles a number of experts with different expertise including the-state-of-art breast cancer PDX models (Lewis), single/few cell RNA-seq (Zong), drug design and synthesis (Song), and biostatistics (Mo). This is expected to generate significant synergy.

5. Changes/Problems

So far, we have not encountered any problems and will stick to our original plan at this point.

6. Products

None so far.

7. Participants & Other Collaborating Organizations

Name:	Quincy Mo
Project Role:	Co-investigator
Researcher Identifier:	N/A
Nearest person month worked	1.2
Contribution	Dr. Mo is responsible for statistical and bioinformatics analyses of our data.
Funding Support	Dr. Mo is also supported by NIH (R01 CA175397, R01 CA072038, R01 CA183964, R01 HD042311), CPRIT (RP120732, RP150129, RP150032), DOD (BC133730 W81XWH-14-1-0378), and Komen foundation (CCR14300500) grants.
Name:	Chenghang Zong
Project Role:	Co-investigator
Researcher Identifier:	N/A
Nearest person month worked	0.6
Contribution	Dr. Zong is an expert of single-cell sequencing, and is helping us establish protocols to sequence BMM transcriptomes, which is critical to delineate molecular mechanisms underlying endocrine resistance of BMM.
Funding Support	Dr. Zong was also supported by NIH New Innovator1DP2EB020399-01
Name:	Yongcheng Song
Project Role:	Co-investigator
Researcher Identifier:	N/A
Nearest person month worked	0.6
Contribution	Dr. Song is an expert of chemical synthesis and modification of drugs. He is helping us to improve bioavailability and pharmacokinetics of potential bone metastasis drugs.

Funding Support	Dr. Song was also supported by NIH R01NS080963, Cancer Prevention and Research Institute of Texas RP140469 and RP150129
Name:	Michael Lewis
Project Role:	Co-investigator
Researcher Identifier:	N/A
Nearest person month worked	0.6
Contribution	Dr. Lewis established a cohort of PDX models. He is helping us utilize PDX models to generate metastasis models for mechanistic and therapeutic studies.
Funding Support	Dr. Lewis is also supported by fundings from NSF (1263742), NIH (CA179720) and Helis Foundation.
Name:	Hai Wang
Project Role:	Research Associate
Researcher Identifier:	N/A
Nearest person month worked	12
Contribution	Dr. Wang specialize in bone metastasis research techniques and is leading the efforts of establishing BICA.
Name:	Igor Bado
Project Role:	Postdoctoral Fellow
Researcher Identifier:	N/A
Nearest person month worked	12
Contribution	Dr. Bado focuses on the role of estrogen receptors in driving bone microenvironment-dependent endocrine resistance.
Name:	Weijie Zhang
Project Role:	Postdoctoral Fellow
Researcher Identifier:	N/A
Nearest person month worked	12
Contribution	Dr. Zhang focuses on the roles of various bone microenvironment niches during bone metastasis colonization.

Name:	Yuande Tan
Project Role:	Postdoctoral Fellow
Researcher Identifier:	N/A
Nearest person month worked	6
Contribution	Dr. Tan worked under the supervision of Dr. Mo, executing statistical and bioinformatic analyses.
Funding Support	Dr. Tan is also supported by BCM internal grant and cancer center support grant CA125123.
Name:	Fangrui Wu
Project Role:	Postdoctoral Fellow
Researcher Identifier:	N/A
Nearest person month worked	6
Contribution	Dr. Wu worked under the supervision of Dr. Song, executing chemical synthesis and modification of epigenomic and FDA-approved drugs that have potential anti-metastasis effects.
Funding Support	Dr. Wu is also supported by CPRIT RP150129.

Name:	Xiang Zhang
Project Role:	PI/PD
Researcher Identifier:	N/A
Nearest person month worked	3.0
Contribution	Dr. Zhang designed and supervised the experiments described in this report.
Funding Support	Dr. Zhang is also supported by NIH/NCI, Breast Cancer Research Foundation, Susan G. Komen Foundation, and McNair Medical Institute.

All collaborators and participants are at Baylor College of Medicine.

8. Special Reporting Requirements

None.

9. Appendices

None.