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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.

This subtask has been accomplished, as reported in previous years.

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).

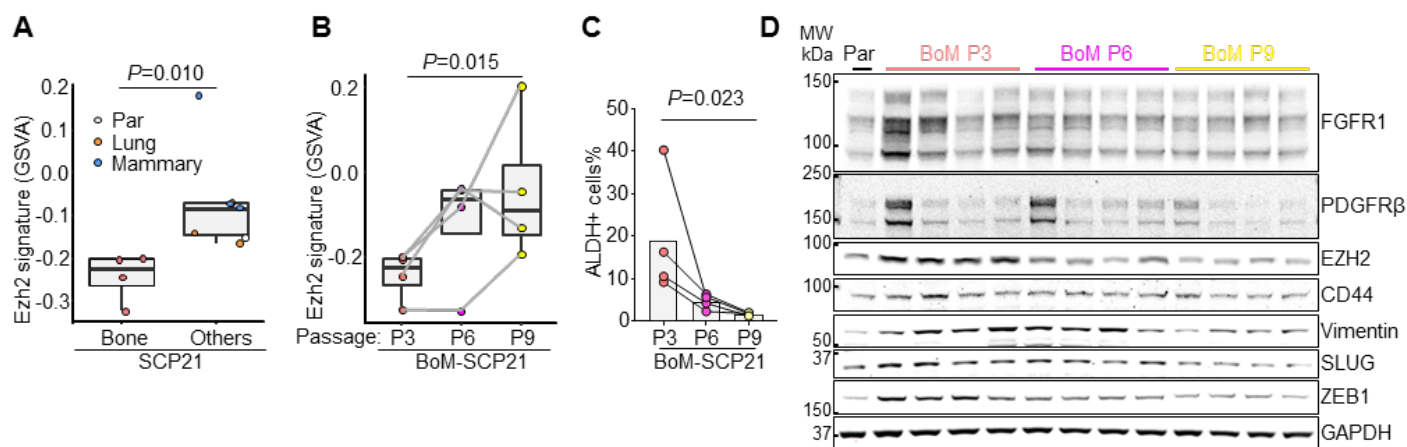


Figure 1. The bone microenvironment reversibly activates EZH2 signaling and increases cancer cell stemness. (A) Levels of EZH2 signature genes (GSVA) in bone entrained and other SCP21 cells. **(B)** Levels of EZH2 signature genes in bone entrained-SCP21 cells after different passages *in vitro*. **(C)** Percentage of ALDH1+ population in bone entrained-SCP21 cells at different passages. **(D)** Representative western blotting of proteins in bone entrained-SCP21 cells after different passages.

In last grant report, we provided detailed analysis uncovering a profound impact of the bone marrow microenvironment on BMMs in MCF-7 cells. In this grant period, we repeated the same experiment on an ER-cell line MDA-MB-231. To distinguish between genetic selection and epigenomic reprogramming, we used a single cell-derived population (SCP) of MDA-MB-231 named SCP21. Specifically, SCP21 cells were transplanted into bone, lungs, and mammary fat pads of host animals. After establishment, the tumors were resected and cancer cells were purified and kept in culture. At different passages, the cells entrained in different organs were compared. Like MCF-7, we observed that an EZH2 signature is markedly suppressed in bone-entrained SCP21 cells as compared to those entrained in mammary glands or lungs (**Fig. 1A**), indicating an enhancement of EZH2 activities (EZH2 typically suppresses gene expression). Interestingly, this alteration seemed to be transient and was progressively lost with more passages in culture (**Fig. 1B**). EZH2 is an important regulator of cancer cell stemness. Consistently, percent ALDH+ cells increased in bone-entrained SCP21 cells (**Fig. 1C**), and so were expression of multiple other proteins related EMT and stemness (**Fig. 1D**). We also observed increase of FGFR1 and PDGFR β , which may be upstream signal transducers of EZH2 (**Fig. 1D**). Importantly, all of these changes are reversible – indicating epigenomic reprogramming rather genetic selection.

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^{f/f} 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).

This subtask has been accomplished, as reported in previous years.

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)

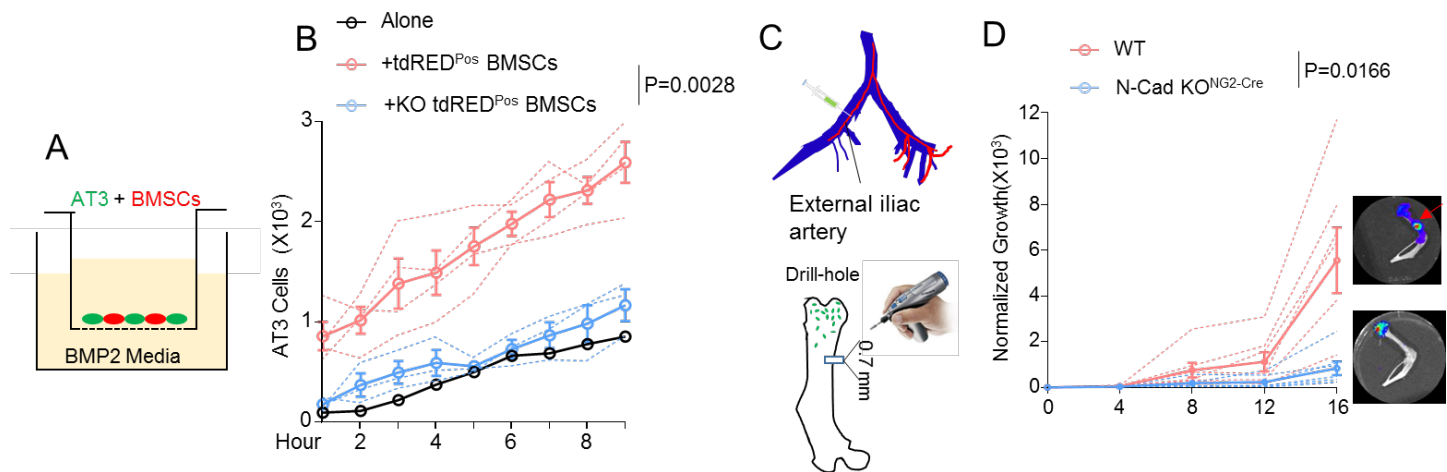


Figure 2. N-cadherin mediates migration by tethering in vitro and in vivo (A) The schematic shows the set up of in vitro MBT assay. (B) Quantitation of AT3 cells that migrate into the bottom chamber as a function of time with or without bone marrow-derived MSCs (BMSCs). KO indicated knockout of N-cadherin. (C) Schematics show intra-iliac artery injection (upper) and drill-hole on tibia bones (lower). (D) Quantitation of bioluminescence intensity of AT3 cells at the drilled site with or without N-cadherin knockout in BMSCs (NG2-cre⁺). Representative bioluminescence pictures are shown for both groups. The red arrow indicates a metastatic lesion at the drilled site.

In last report, we described our discovery that cancer cells can adhere to and migrate together with osteogenic cells. This phenomenon was named “migration-by-tethering” or MBT. MBT allows cancer cells to relocate from the perivascular niche to osteogenic niche, which may terminate dormancy¹. N-cadherin may provide the physical basis for the two cell types to stick together. In this grant period, we validated this hypothesis in vitro and in vivo.

In vitro, we set up a modified trans-well migration assay to test MBT. In the upper level of the Boyden chamber, GFP-tagged AT3 breast cancer cells and bone-derived MSCs (BMSCs) were intermixed. BMSCs were genetically tagged with tandem repeat RFP (tdRED). In the lower chamber, we added osteogenic signal, BMP2 (Fig. 2A). Without BMSCs, AT3 cells do not respond to BMP2. However, the presence of BMSCs allows AT3 to migrate across the well. We have shown in our published work that this occurs through MBT². Here, we compared BMSCs with or without N-cadherin knockout (KO) and observed that KO almost completely abolished MBT, supporting the essential role of N-cadherin in MBT (Fig. 2B).

In vivo, we used a bone-drilling setting to activate bone remodeling and BMSC migration toward the osteogenic site. Specifically, we first transplant AT3 cells into bone through external iliac-artery. One week after the transplantation, we drilled a hole on tibia bone to introduce bone fracture (Fig. 2C). In previous report, we showed that N-cadherin knockout delayed bone healing. Here, we further observed that lack of N-cadherin also prevents cancer cells from moving to the bone remodeling site to establish ectopic bone lesions (Fig. 2D). Together with results previous reported, this data strongly support an important function of N-cadherin of MsCs during bone colonization, especially in enabling MBT and terminating dormancy.

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)

This subtask has been finished, and results were updated in previous reports.

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).

This subtask has been finished, and results were updated in previous reports.

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)

This subtask has been finished, and results were updated in previous reports.

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)

This subtask has been accomplished, as reported in previous years.

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)

This subtask has been accomplished, and several new findings have been made as reported before.

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

This effort has started and generated promising outcome. Representative results are shown in **Fig. 3**. We will collectively report the summary of all relevant results in the final report.

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)

This subtask has been accomplished, as reported in previous years.

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

This subtask has been accomplished and reported last year.

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

Last year, we reported our collaboration with Dr. Han Xiao's laboratory at Rice University to conjugate chemical drugs with therapeutic antibodies to achieve better efficacies and specificities against DTCs and BMMs. Dr. Xiao pioneered a technology named pClick, a new antibody conjugation approach. We reported a study to demonstrate the improved efficacies of bisphosphonate-conjugated anti-Her2 treatment on Her2+ bone metastases. Interestingly, in the past grant period, we found that even Her2- cancer cells may overexpress Her2 in the bone marrow microenvironment, which makes anti-Her2 treatment potentially applicable to these tumors too. Indeed, a proof-of-principle experiment was performed using MCF7 cells (ER+Her2-). We observed significant efficacy of bisphosphonate-conjugated trastusumab (**Fig. 3A-3E**). Notably, upregulation of Her2 also occurs in 2/3 additional ER+ breast cancer models including a PDX (**Fig. 3F**). We are now pursuing this finding mechanistically and therapeutically.

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)

We have made significant progress and uncovered several intriguing mechanisms underlying cancer-bone interactions and therapeutic resistance of bone metastases. These mechanisms have been reported in previous reports. We will summarize them in our final report.

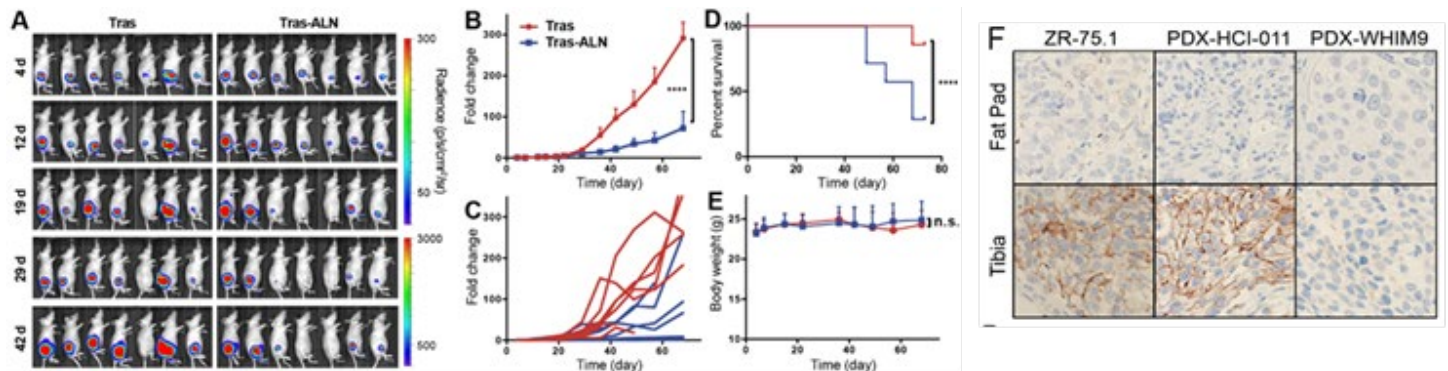


Figure 3. In vivo comparison of Tras and Tras-ALN in HER2-negative model. (A) Tumor burden was monitored by weekly bioluminescence imaging, and (B) quantified by the radiance detected in the region of interest. (C) Fold-change in individual luminescent intensity of HER2-negative MCF-7 tumors in mice treated as described in (A). (D) Kaplan-Meier plot of the time-to-sacrifice of mice treated as described in (A). (E) Body weight change of tumor-bearing mice over time. **** $P < 0.0001$ and n.s. = $P > 0.05$. (F) IHC of Her2 in three ER+Her2- models transplanted either into mammary tumors or tibia bones.

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), and drug design and synthesis (Song). This is expected to generate significant synergy.

The research outcomes from the first four grant years include the discovery of novel mechanisms underlying cancer-bone interactions and identification of potential novel therapies that may help eliminate bone micrometastases and prevent overt recurrences.

5. Changes/Problems

The Covid 19 pandemic has significantly impacted our ability to access lab and animal facilities. However, we have still made significant progress.

6. Products

Publications:

1. Bado IL, Zhang W, Hu J, Xu Z, Wang H, Sarkar, Li L, Wan Y-W, Liu J, Wu W, Lo H-C, Kim IS, Singh S, Janghorban M, Muscarella AM, Goldstein A, Singh P, Jeong H-H, Liu C, Schiff R, Huang S, Ellis MJ, Gaber MW, Gugala Z, Liu Z, Xiang H.-F. Zhang (2021) Impact of the bone microenvironment on phenotypic plasticity of ER+ breast cancer cells. Developmental Cell, In Press.

2. Zhang W, Bado IL, Hu J, Wang Y-W, Wu L, Wang H, Gao Y, Jeong H-H, Xu Z, Hao X, Lege BM, Al-Ouran R, Li L, Li J, Yu L, Singh S, Lo H-C, Niu M, Liu J, Jiang W, Li Y, Wong STC, Cheng C, Liu Z, and Xiang H.-F. Zhang (2021). The bone microenvironment invigorates metastatic seeds for further dissemination. Cell, In Press.
3. Muscarella AM, Dai W, Mitchell PG, Zhang W, Wang H, Jia L, Stossi F, Mancini MA, Chiu W, and Xiang H.-F. Zhang (2020) “Unique cellular protrusions mediate breast cancer cell migration by tethering to osteogenic cells”, npj Breast Cancer, Published online: 09-07-2020.
4. Muscarella AM, Aguirre S, Hao X, Waldvogel SM, Xiang H.-F. Zhang. Exploiting bone niches: progression of disseminated tumor cells to metastasis. Journal of Clinical Investigation 2021 Mar 15;131(6):e143764. doi: 10.1172/JCI143764.
5. Tian Z, Wu L, Yu C, Chen Y, Xu Z, Bado I, Loredó A, Wang L, Wang H, Wu K-L, Zhang W, Xiang H.-F. Zhang*, and Han Xiao*. *Harnessing the Power of Antibodies to Fight Bone Metastasis*. Science Advances. Accepted. (* co-corresponding authors)

Grants (pending):

1. DoD BCRP BC201371P1 Eliminating Bone Micrometastases by Bone-targeting Antibodies

7. Participants & Other Collaborating Organizations

Name:	Chenghang Zong
Project Role:	Co-investigator
Researcher Identifier:	N/A
Nearest person month worked	0.36
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:	Instructor
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:	Yang Gao
Project Role:	Postdoctoral Fellow
Researcher Identifier:	N/A
Nearest person month worked	6
Contribution	Dr. Yang focuses on the role of estrogen receptors in driving bone microenvironment-dependent endocrine resistance.
Name:	Zhan Xu
Project Role:	Postdoctoral Fellow
Researcher Identifier:	N/A
Nearest person month worked	12
Contribution	Dr. Xu focuses on the roles of various bone microenvironment niches during bone metastasis colonization.

Name:	Xiang Zhang
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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, and McNair Medical Institute.

All collaborators and participants are at Baylor College of Medicine.

8. Special Reporting Requirements None.

9. Appendices

A copy of the publications or editorial acceptance letters.

REFERENCES

- 1 Muscarella, A. M., Aguirre, S., Hao, X., Waldvogel, S. M. & Zhang, X. H. Exploiting bone niches: progression of disseminated tumor cells to metastasis. *J Clin Invest* **131**, doi:10.1172/JCI143764 (2021).
- 2 Muscarella, A. M. Unique cellular protrusions mediate breast cancer cell migration by tethering to osteogenic cells. *npj Breast Cancer*, doi:10.1038/s41523-020-00183-8 (2020).

Zhang, Xiang

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Dr. Xiang Zhang

The bone microenvironment increases phenotypic plasticity of ER+ breast cancer cells
Manuscript Number: DEVELOPMENTAL-CELL-D-21-00166R2

Feb 27, 2021

Dear Shawn,

Thank you for making the final adjustments to the manuscript. I am happy to accept your manuscript for publication in *Developmental Cell*. Congratulations on the paper! We are delighted to have the opportunity to present these exciting findings to the scientific community.

Your paper is currently being scheduled for the April 19th issue (and we are coordinating with Cell).

At this time, I also wanted to introduce our Production Editor, Karen Helgeson, who heads up the production team that will guide your paper through copyediting, proofing, and publication. Karen Helgeson will be contacting you shortly with some initial information on the production process. If you have any questions about this process, please email khelgeson@cell.com.

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Congrats again on the paper, and thank you for sending it to *Developmental Cell*. I look forward to seeing its impact on the field. I also hope you have been happy with the publishing process at *Developmental Cell*, and will consider us again in the future as a venue for your best work.

Kind regards,

Ilil

Ilil Carmi, Ph.D.
Scientific Editor, *Developmental Cell*

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Dr. Xiang Zhang
Baylor College of Medicine
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One Baylor Plaza, BCM600
Houston, Texas 77030
UNITED STATES

The bone microenvironment invigorates metastatic seeds for further dissemination
CELL-D-19-02641R3

Mar 04, 2021

Dear Xiang,

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Unique cellular protrusions mediate breast cancer cell migration by tethering to osteogenic cells

Aaron M. Muscarella^{1,2,3,4}, Wei Dai⁵, Patrick G. Mitchell^{4,6,7,8}, Weijie Zhang^{1,2,3}, Hai Wang^{1,2,3}, Luyu Jia⁴, Fabio Stossi^{2,3}, Michael A. Mancini^{2,3}, Wah Chiu⁸ and Xiang H.-F. Zhang^{1,2,3,9}

Migration and invasion are key properties of metastatic cancer cells. These properties can be acquired through intrinsic reprogramming processes such as epithelial-mesenchymal transition. In this study, we discovered an alternative “migration-by-tethering” mechanism through which cancer cells gain the momentum to migrate by adhering to mesenchymal stem cells or osteoblasts. This tethering is mediated by both heterotypic adherens junctions and gap junctions, and leads to a unique cellular protrusion supported by cofilin-coated actin filaments. Inhibition of gap junctions or depletion of cofilin reduces migration-by-tethering. We observed evidence of these protrusions in bone segments harboring experimental and spontaneous bone metastasis in animal models. These data exemplify how cancer cells may acquire migratory ability without intrinsic reprogramming. Furthermore, given the important roles of osteogenic cells in early-stage bone colonization, our observations raise the possibility that migration-by-tethering may drive the relocation of disseminated tumor cells between different niches in the bone microenvironment.

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INTRODUCTION

Metastasis is the major challenge in research and treatment of cancers^{1–3}. The metastasis cascade involves cellular migration. This is intuitively obvious as cancer cells are expected to leave primary tumors and enter distant organs, and almost every step of this process requires mobility. Multiple cell-intrinsic migratory mechanisms can be activated by epithelial-mesenchymal transition (EMT), during which cancer cells lose epithelial traits and gain motility⁴. More recently, a basal-like reprogramming process has been observed to render cancer cells migratory without losing cell-cell adhesions, also known as “collective migration”^{5–7}. Both EMT and collective migration can be induced by the microenvironment through paracrine or direct cell-cell contact. In particular, a recent study demonstrated that cancer-associated fibroblasts (CAFs) can lead collective migration through development of heterotypic adherens junctions constituted by N-cadherin of CAFs and E-cadherin of cancer cells⁸. These findings provide novel insights into the escape of tumor cells from primary tumors.

After disseminated tumor cells (DTCs) enter distant organs such as bone, cellular migration may still be required before full colonization is accomplished. Increasing evidence suggests that the microenvironment in distant organs is compartmentalized into various niches. The unique cellular components and extracellular matrix of each niche determine the fate of cancer cells⁹. The perivascular niche, for instance, has been shown to maintain dormancy of DTCs^{10,11}. On the other hand, we and others elucidated that the osteogenic niche promotes proliferation and early-stage bone colonization^{12–14}. Thus, relocation between different microenvironment niches may represent one mechanism to switch cellular fates of DTCs. This relocation may involve a

distinctive type of migration, which occurs when cancer cells exist as single- or few-cell micrometastases and are fully surrounded by various normal cells – a situation in contrast to what is known for migration and invasion in primary tumors. The cellular/molecular nature of this type of migration is completely elusive.

Here, we observed that proximity of cancer cells to osteogenic cells (e.g., mesenchymal stem cells and osteoblasts) increases the mobility of the former, and allows the otherwise inert cancer cells to move toward osteogenic signals. This process is mediated by a dendritic spine-like structure (DSLS) of cancer cells, which physically attaches to osteogenic cells through heterotypic adherens junctions and gap junctions. DSLS is supported by actin filaments coated with cofilin, and therefore, is highly pliable. Both gap junctions and cofilin are necessary for the maintenance of DSLS and for this “migration-by-tethering” mechanism. Evidence of DSLS *in vivo* was also obtained and suggests that the “migration-by-tethering” may represent a unique migration process of DTCs in the bone microenvironment.

RESULTS

Cancer cells gain mobility through a MBT mechanism in co-culture with osteogenic cells

Our previous studies have established a central role of osteogenic cells (the cells in the osteogenic lineage from MSCs to newly developed osteoblasts) in early-stage bone colonization^{12,13}. These cells constitute the osteogenic niche and support cancer cell proliferation through adherens junctions and gap junctions. Cancer cells and osteogenic cells readily establish direct cell-cell contact *in vivo* and in 3D suspension co-cultures (Supplementary

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Fig. 1a), making us hypothesize chemotaxis between the two cell types. To test this hypothesis, we set up a simple two-chamber culture system as illustrated in Fig. 1a. The chamber walls can be removed to allow originally separated cells to interact or migrate towards the other chamber. We started with MCF-7 cells because these ER+ breast cancer cells can home to the osteogenic niche in vivo and form extensive cell-cell contact with osteogenic cells in 3D co-cultures as we showed in our previous works¹².

Inconsistent to our hypothesis, MCF-7 cells did not exhibit chemotactic movement toward osteogenic cells in the adjacent chamber (Fig. 1b, c). In fact, MCF-7 cells maintain epithelial traits and are poorly migratory: when cultured alone, very few cells could move across the border between the chambers (Fig. 1b and Supplementary Video 1). However, when they were directly admixed with osteoblasts in the same chamber, the frequency of MCF-7 cells that could migrate across chambers evidently increased (Fig. 1b, c). Real-time imaging revealed that cancer cells stick to the much more mobile osteogenic cells and are “dragged” to move (Supplementary Fig. 1b and Supplementary Video 2), a phenomenon that we termed “migration-by-tethering” (MBT). We next asked if other major cell types in the bone microenvironment can also drive MBT. Human mesenchymal stem cells (hMSCs) and MC3T3-E1 pre-osteoblasts cells showed this ability when co-cultured with MCF-7. However, RANK-L differentiated Raw 264.7 osteoclasts failed to increase of MCF-7 motility (Fig. 1d). Thus, it suggests that, for cancer cells in the bone, MBT can be mediated by cells with osteogenic potential (i.e., osteogenic cells).

To examine cancer cell specificity related to MBT, we tested several more human and murine cancer cell lines known to establish bone metastases according to previous studies, namely 4T1, 4T1.2, AT3, and SCP28^{13,14}. All of these lines exhibited similar MBT properties (Fig. 1e, f and Supplementary 1c). In particular, SCP28 is a single cell-derived, bone-seeking subpopulation of MDA-MB-231¹⁵. One difference between SCP28 and the parental population is the expression of E-cadherin (Supplementary Fig. 1d). Interestingly, migration of MDA-MB-231 parental cells did not increase when co-cultured with osteogenic cells (Fig. 1e), raising the possibility that E-cadherin is involved in MBT. In fact, other cell lines with MBT capacity are also E-cadherin positive as we previously showed (Fig. 1f)¹². The potential role of E-cadherin in MBT will be further discussed in a later section.

In physiological conditions, osteogenic cells respond to osteogenic signals by chemotaxis and differentiation. Our initial observations suggest that cancer cells can be attracted to sources of osteogenic signals through MBT. We tested this possibility by transwell assays in which cancer cells and/or osteogenic cells are admixed in the upper chamber and BMP-2-containing media is placed in the bottom chamber. As expected, transwell migration of MCF-7 cells was changed very little by BMP-2 when they are cultured alone, but significantly increased when osteogenic cells are co-cultured (Fig. 1g and Supplementary Fig. 1e)—supporting that osteogenic cells and MBT may drive relocation of cancer cells toward sites releasing osteogenic signals. While direct treatment of BMP-2 can increase MCF-7 migration through transwell¹⁶, in this experiment, with cells at relatively low density and with BMP-2 used as a chemoattractant, no statistically significant difference was recorded. This increase is not due to proliferation, as proliferation inhibitors did not reduce the number of cells that cross the membrane when osteoblasts and BMP-2 are present (Supplementary Fig. 1f). Further experiments suggested that the increase in migration is osteoblast-contact-dependent, not a paracrine attraction, as osteoblasts in the bottom chamber of the transwell insert do not attract cancer cells on their own (Supplementary Fig. 1g). Additionally, we also excluded the possibility that this increase is due to extracellular matrix deposited by osteoblasts. When osteoblasts were removed with EDTA instead of trypsin, leaving osteoblast-produced ECM, the subsequently added cancer cells migrate notably less (Supplementary Fig. 1h),

suggesting that live osteoblasts are the major driving force of the increased transwell migration toward osteogenic signals.

Cancer cells adhere to osteogenic cells via DSLS

We then set out to understand the cellular and molecular mechanisms underlying MBT. Higher-resolution, real-time microscopy revealed an interesting process: a long cellular protrusion was stretched out from cancer cells by osteoblasts that are migrating. This protrusion elongated as osteoblasts moved apart and maintained the direct cell-cell contact. In many cases, the protrusion was finally dissociated from osteogenic cells and regressed (Fig. 2a and Supplementary Video 3). In some cases, the connection eventually led to co-migration of cancer cells in an abrupt manner (Fig. 2b, Supplementary Videos 4 and 5). Through deconvolution microscopy, we observed that the protrusion structure morphologically resembles dendritic spines of neurons^{17,18}—a small membranous protrusion from a neuron's dendrite that typically receives input from a single axon at the synapse. It comprises of a thin neck that can be as long as 100 μm and usually with a spherical head at the terminal (Fig. 2c). Sometimes additional spherical structures can be found in the middle of the neck. Therefore, we named this protrusion dendritic spine-like structure, or DSLS.

We next used cryo-electron tomography (cryo-ET) to characterize DSLS at nm resolution, and confirmed that DSLS physically contacts osteogenic cells, and at that contact point there is an altered organization of the cytoskeleton (Fig. 2d). When cancer cells are not in proximity to osteogenic cells, shorter “buds” of DSLS could be observed at cell periphery, which are clearly distinct from typical filopodia, with regards to size and actin composition (filopodia contain actin bundles)¹⁹ (Fig. 2e). However, elongated DSLS increased when cancer cells were co-cultured with osteogenic cells, but not other bone cell types including fibroblasts and osteoclasts (Fig. 2f). We also observed DSLS in multiple other cancer cell lines including some non-breast cancers, suggesting that this is not a breast cancer-specific phenomenon (Supplementary Fig. 2a and Supplementary Table 1).

The morphological features distinguish DSLS from other previously described sub-cellular structures such as migrasomes²⁰, tunneling nanotubes²¹ and cytonemes²². Migrasomes are derived from retraction fibers that trail behind migrating cells, and eventually are deposited behind the cells²⁰. This is the opposite to DSLS, which leads the way of migration. We observed tunneling nanotubes between AT3 cells in their monocultures, usually in the form of multiple parallel tubes (Supplementary Fig. 2b). These tubes are not apparently associated with migration and morphologically distinct from DSLS connecting the cancer cells to osteoblasts. The expression of myosin X, a key component of cytonemes, did not change in co-cultures with osteoblasts (Supplementary Fig. 2c).

DSLS is also distinct from filopodia and lamellipodia, as indicated by its unique responses to actin/microtubules perturbations. While actin assembly inhibitor latrunculin B was shown to decrease filopodia formation²³, this treatment increased DSLS development (Supplementary Fig. 2d). Jasplakinolide, an actin stabilizer and promotor of nucleation, was shown to decrease lamellipodia²⁴, but again increased DSLS frequency (Supplementary Fig. 2d). The effect size of these two agents is ~3–5 fold by manual counting. Furthermore, nocodazole, a microtubule polymerization blocker, increased filopodia density²⁵, but had no clear effect on DSLS prevalence or structure (Supplementary Fig. 2d). Taken together, DSLS appears to be distinctive from the previously characterized cellular protrusions.

Cofilin is an essential cytoskeletal component of DSLS and necessary for MBT

We performed additional cryo-ET to visualize possible cytoskeleton supporting DSLS. Strand structures were observed in the neck of DSLS, suggesting actin filaments (Fig. 3a, b), which was

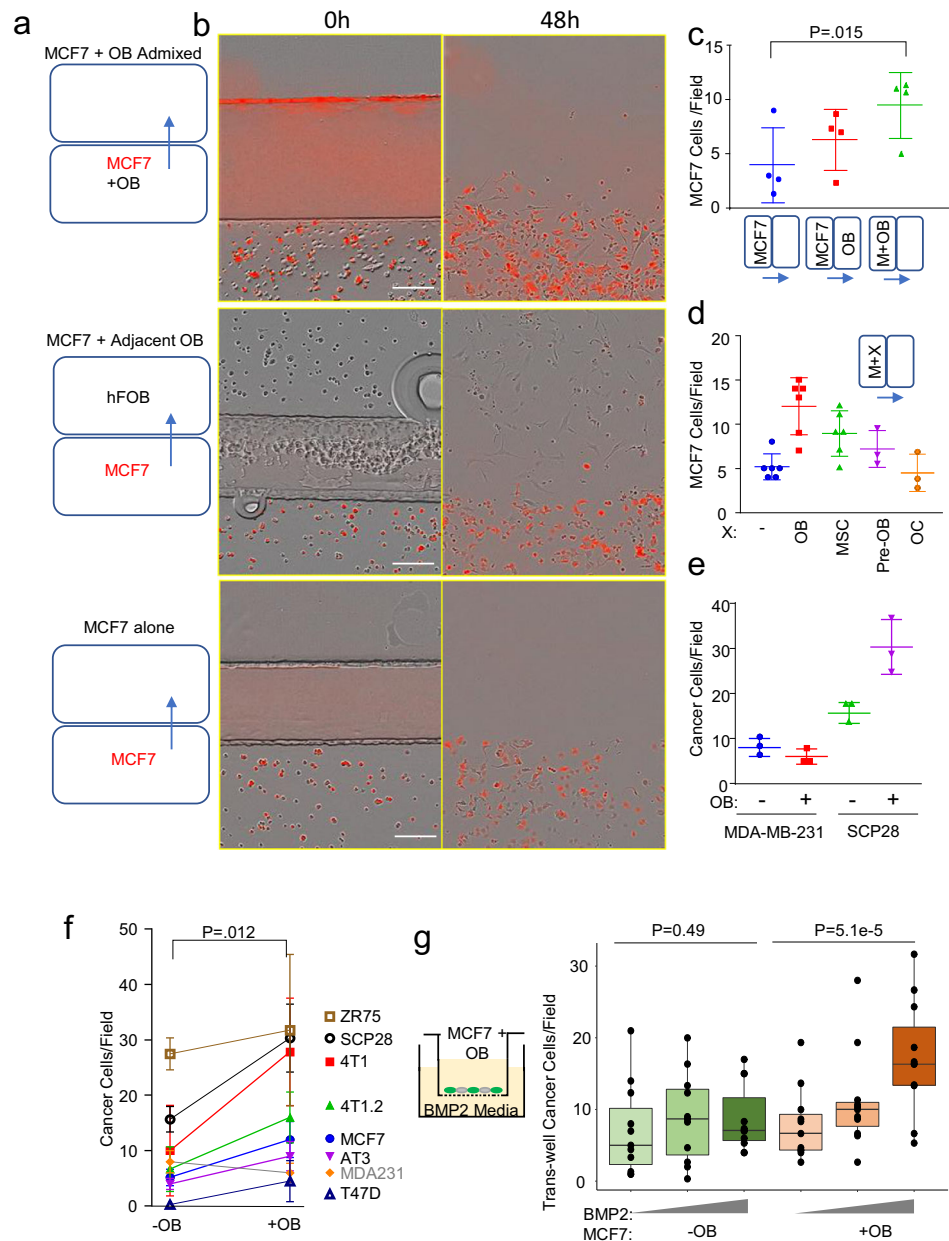


Fig. 1 Breast cancer cells which express E-cadherin have increased migration when cultured with osteogenic cells in vitro. **a** Ibidi silicone removable chambers were used to quantify migration by breast cancer cells in monoculture, in co-culture with osteoblasts in adjacent chambers, or in direct admixture in the same chamber. **b** To distinguish cell types, fluorescently labeled breast cancer cells were co-cultured with unlabeled osteoblasts (OBs), and imaged with the Incucyte S3. Scale bar is 100 μ m. **c** Dot plot showing the number of cells escaped from their original enclosure per field for MCF-7 alone, in co-culture with OBs in adjacent regions, or in co-culture with osteoblasts in the same region after 48 h. P -value determined by students t -test (two-tailed). Each dot represents a biological replicate, an average of three technical replicates. **d** Dot plot shows the number of cells escaped per field for MCF-7 alone, and in co-culture with OBs (represented by hFOB1.19 cells), pre-osteoblasts (Pre-OB represented by MC3T3-E1 cells), human mesenchymal stem cells (MSCs), and osteoclasts (OC RANK-L differentiated Raw 264.7 cells) after 48 h. Each dot represents a technical replicate. **e** Dot plot shows the number of cells escaped per field for mesenchymal MDA-MB-231 alone and with osteoblasts, as well as MDA-MB-231 E-cadherin expressing subline SCP-28 alone and with osteoblasts after 48 h. Each dot represents a technical replicate. **f** Box plot shows the transwell migration (a diagram shown in the left) of MCF-7 cells toward a gradient (0, 1, and 5 μ M) of Bone Morphogenetic Protein-2 (BMP-2) with or without admixture of OBs. Each dot represents a biological replicate generated from an independent experiment average of three technical replicates. For **c–f** error bars indicate standard deviation. (For **g**), the lower and upper hinges correspond to the first and third quartiles (the 25th and 75th percentiles). The upper whisker extends from the hinge to the largest value no further than $1.5 \times \text{IQR}$ from the hinge. The lower whisker extends from the hinge to the smallest value at most $1.5 \times \text{IQR}$ of the hinge. Data beyond the end of the whiskers are called “outlying” points and are plotted individually. P -value as determined by analysis of covariance (ANCOVA) as elaborated in “Methods”.

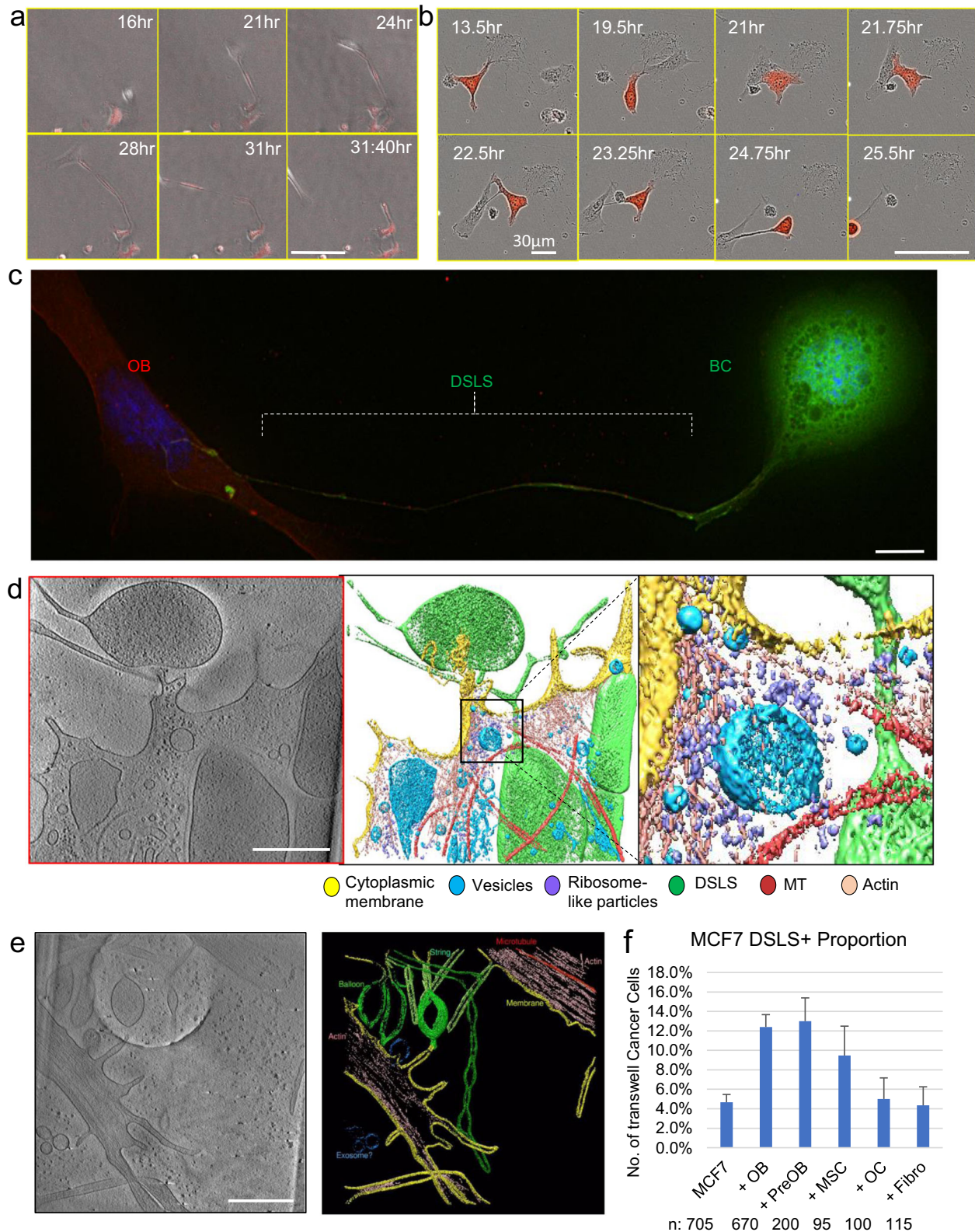
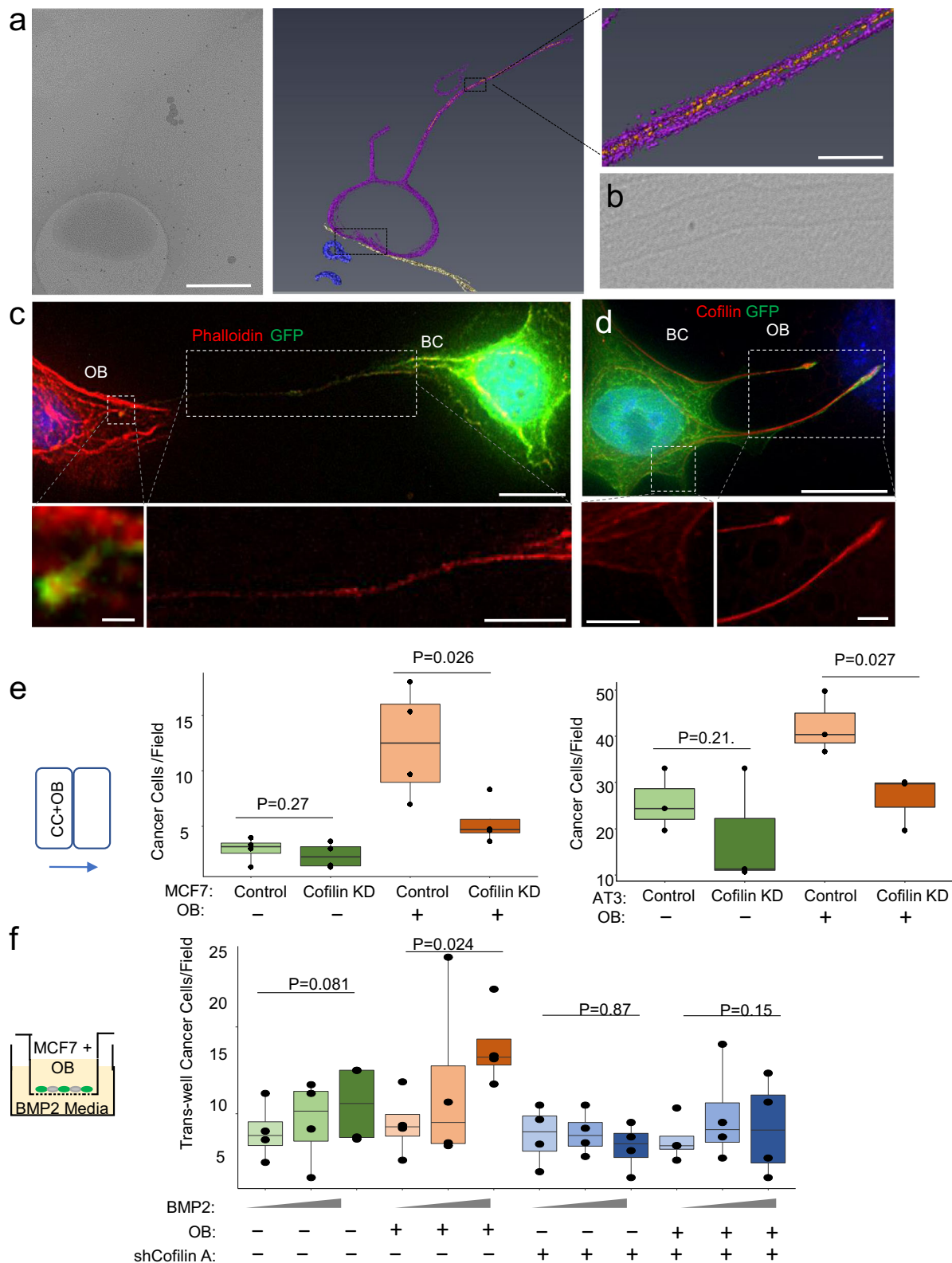


Fig. 2 Luminal breast cancer cells form dendritic spine like protrusions that tether them to mobile osteoblasts. **a** Time-lapse on Cytation of mobile osteoblasts contacting and drawing out cellular protrusion from MCF-7 (red). Scale bar is 100 μ m. **b** Time-lapse on Incucyte S3 of osteoblast (unlabeled) drawing out cellular tether protrusion that leads to MCF-7 (RFP-labeled) migration. Scale bar is 100 μ m. **c** Deconvolution imaging of MCF-7 cell (green) with dendritic spine-like structures (DSLS) protrusions attached to OBs (red). Scale bar is 10 μ m. **d** Tomogram of the terminus of DSLS (green) touching OB cell membrane (yellow). Detail of actin and ribosome concentration where cells make contact. Scale bar is 500 nm. **e** Tomogram of multiple DSLS structures and actin within. Scale bar is 500 nm. **f** The relative abundance of MCF-7 DSLS structures in co-culture with various bone cells, normalized to abundance in monoculture. OB: osteoblasts represented by hFOB 1.19 cells. PreOB: Pre-osteoblasts represented by MC3T3-E1 cells. OC: osteoclasts represented by RAW 264.7 cells. MSC: immortalized Mesenchymal Stem cells Fibro: fibroblasts represented by 293T cells. Error bars indicate standard deviation calculated based on binomial distribution (see “Methods”).



confirmed by deconvolution microscopy and phalloidin staining (Fig. 3c). The pliability of DSLS led us to suspect that the actin filaments must be associated with other proteins that confer flexibility. Cofilin is a compelling candidate because it is known to sever actin filaments to allow bending^{26–28}. Immunofluorescence

staining confirmed this hypothesis. Indeed, cofilin was abundantly expressed along the long axis of DSLS and aligned well with actin filament (Fig. 3d). This distribution provides an explanation for the remarkable pliability of DSLS, and also distinguishes DSLS from regular filopodia in which cofilin is organized in parallel with

Fig. 3 **Protrusions are driven by actin, cofilin, adhere to OBs using E-Cadherin.** **a** Cryo-ET tomogram showing actin fibers (orange) within the stalk of MCF-7 DSLS (purple) contacting an osteoblast (yellow). Scale bars are 500, 50 nm, respectively. **b** Micrograph from an additional MCF-7 DSLS showing actin fibers. **c** Phalloidin staining shows actin filaments in the DSLS stalk. Scale bars are 15 (upper), 1 (lower left), and 10 μ m (lower right) respectively. **d** Cofilin immunolabeling of DSLS. The red channel intensity is matched for both the zoom of DSLS and of the cell body, normal filopodia to emphasize cofilin distribution. Scale bars are 15 (upper), 5 (lower left), and 5 μ m (lower right), respectively. **e** Box and dot plots showing the cells per field migrated out of the chamber for MCF-7 and AT3 cells transfected with GIPZ shRNA construct Empty Vector (Control), and a shRNA against cofilin for knockdown (Cofilin KD), with and without OBs. Each dot represents a biological replicate, an average of three technical replicates. **f** Box plot showing the transwell migration cells through the membrane per field for MCF-7 transfected with GIPZ shRNA construct Empty Vector, and the shRNA against cofilin (Cofilin KD) with and without OBs. Each dot represents a biological replicate, an average of three technical replicates. For box plots, the lower and upper hinges correspond to the first and third quartiles (the 25th and 75th percentiles). The upper whisker extends from the hinge to the largest value no further than $1.5 \times$ IQR from the hinge. The lower whisker extends from the hinge to the smallest value at most $1.5 \times$ IQR of the hinge. Data beyond the end of the whiskers are called “outlying” points and are plotted individually. *P*-value as determined by either ANOVA (**e**) or ANCOVA (**f**) as elaborated in Methods.

membrane but perpendicular to direction of elongation (Fig. 3d, left inset). Expression of eGFP::cofilin fusion proteins confirmed that cofilin is present in the neck of DSLS, and enriched into small bands immediately adjacent to areas of increased flexibility of the structure (Supplementary Fig. 3a and Supplementary Video 6). The interaction between cancer cells and osteogenic cells modestly enhanced transcription of cofilin-encoding gene. (Supplementary Fig. 3b). To examine if cofilin is required for MBT, we knocked down (KD) cofilin by shRNAs in MCF-7 cells (Supplementary Fig. 3c). As expected, although cofilin KD did not affect MCF-7 migration in monocultures, it reduced the frequencies of prolonged DSLS (Supplementary Fig. 3d) and decreased co-migration of MCF-7, AT3, and 4T1 cells with osteogenic cells (Fig. 3e and Supplementary Fig. 3e). Consistently, chemotaxis of osteogenic cells toward BMP-2, the osteogenic signal, drives transwell migration of MCF-7 and 4T1 cells in a cofilin-dependent manner, supporting the MBT mechanism (Fig. 3f and Supplementary Fig. 3f). Of note, the degree of knockdown of cofilin did not seem sufficient to block migration of MCF-7 cells toward EGF (Supplementary Fig. 3g). Thus, the role of cofilin in MBT toward osteogenic signals appears to be more pronounced as compared to that in general migration. The phosphorylation status of cofilin is crucial to its function, with phosphorylated cofilin being the inactive form, with a variety of upstream mechanisms of regulation²⁹. For this reason, we performed western blot and immunofluorescence staining of p-cofilin (Supplementary Fig. 3h, i). The signal was extremely weak in both assays, and appeared to be similar between monoculture vs coculture with osteoblasts. This may be consistent with p-cofilin being unbound to actin and dispersed in the cytoplasm²⁶. Profilin, another small actin-binding protein involved in actin-polymerization dynamics, is prominently expressed by MCF-7 cells, but there is little change in profilin expression between monoculture and coculture, or when cofilin is knocked down (Supplementary Fig. 3j)³⁰.

Gap junctions between DSLS and osteogenic cells are necessary for DSLS maintenance and MBT

Bone micrometastases develop heterotypic adherens junctions (hAJs) and gap junctions (GJs) with osteogenic cells when they are in full contact^{12,13}. Here, we asked whether the physical contact between DSLS and osteogenic cells is also mediated via hAJs and GJs, before more extensive cancer-osteogenic cell contact is established. Immunofluorescence staining revealed that E-cadherin is often concentrated at the head of DSLS (Fig. 4a), and co-localizes with N-cadherin (Fig. 4b).

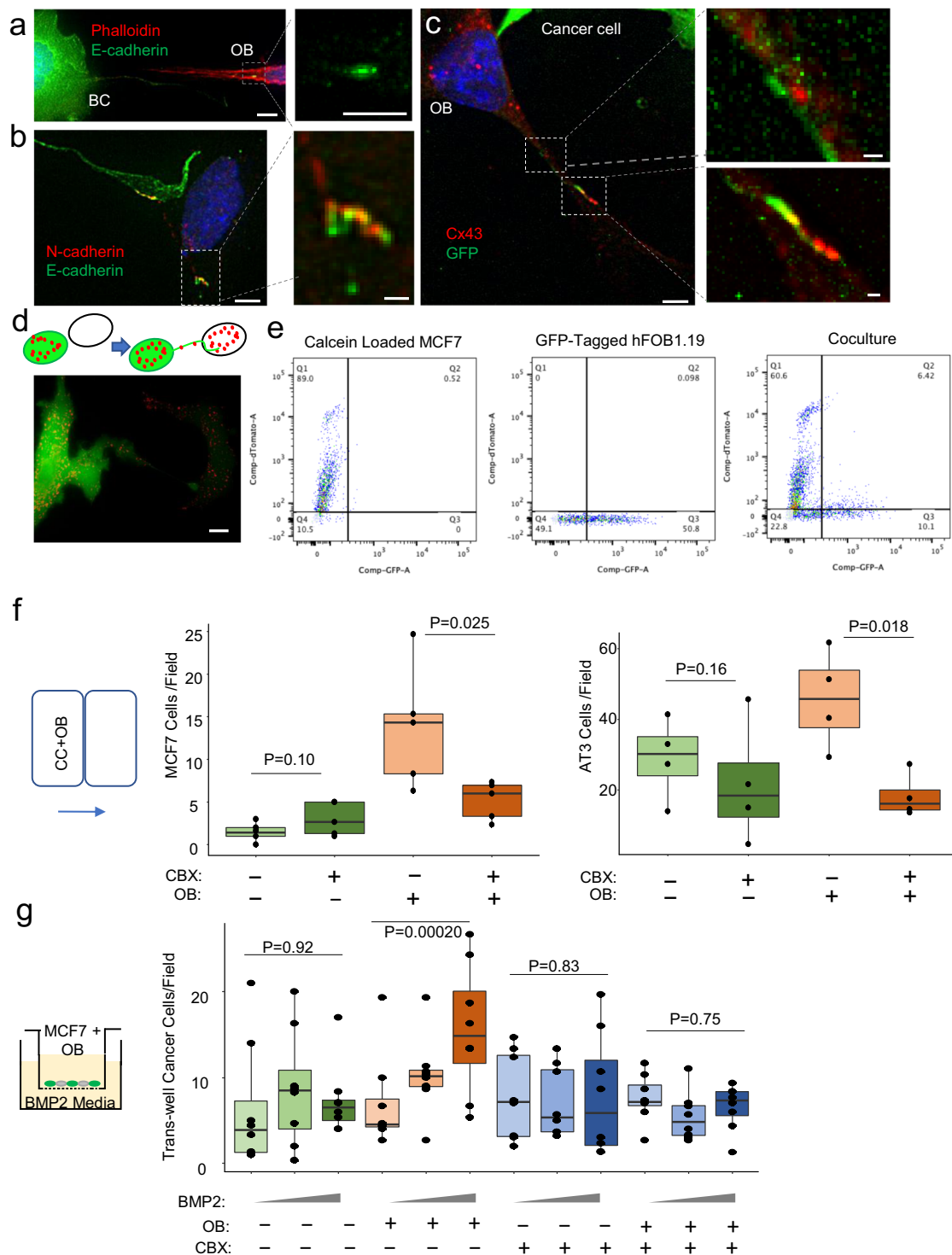
Adherens junctions often precede the development of, and can stabilize GJs, which allow direct substance diffusion between the contacting cells^{31–33}. Connexin 43 (Cx43), a major component of GJ in bone micrometastases¹³, was expressed at the interface between DSLS and osteogenic cells (Fig. 4c). Pre-incubation of cancer cells with calcein, a small fluorescence dye that can diffuse through GJ, clearly indicated GJ functions between cancer cells

and osteogenic cells as shown by live cell fluorescence imaging and flow cytometry (Fig. 4d, e). We asked if GJs are necessary for DSLS development and MBT. Inhibition of GJ by an inhibitor, carbenoxolone (CBX), significantly decreased DSLS (Supplementary Fig. 4a) and reduced MBT in the 2D chamber and osteogenic transwell assay (Fig. 4f, g and Supplementary Fig. 4b). Similarly, a Cx43-specific inhibitory peptide, Gap19, achieved comparable effects and specifically block MBT in 2D and transwell settings (Supplementary Fig. 4c). Notably, the reduction of MBT is not due to the decrease of osteoblast migration (Supplementary Fig. 4d). Again, to address the specificity of BMP-2 and to distinguish MBT from cancer cell-intrinsic migration mechanisms, we also performed a similar experiment using EGF to replace BMP2 and found no significant reduction of EGF-induced cell migration from CBX (Supplementary Fig. 4e). To specifically disrupt Cx43 on either cell type, we transduced cancer cells with a dominant-negative mutant of Cx43, and transfected osteoblasts with Cx43-targeting siRNA before co-culture, respectively. Both treatments significantly decreased BMP-2-induced MBT (Supplementary Fig. 4f). Additionally, cofilin knockdown alone did not affect Cx43-expression by the cancer cells, indicating that Cx43 was independently important and not a downstream effect of cofilin loss (Supplementary Fig. 4g). These results support that the previously observed differences are indeed caused by inhibition of MBT but not general motility of cancer cells.

In vivo evidence supporting the existence of DSLS in bone metastases

We asked if DSLS can be observed in bone lesions. Several difficulties were encountered. First, the morphologically distinct neck of DSLS in 3D tissues may be too long and thin to be completely visualized by a 2D focal plane, even when imaging a large field of view by microscopy. Second, MBT is transient. Hence, the time window to visualize this process may be narrow, requiring a large number of incidents to be monitored. Third, bone is a calcified tissue and highly auto-fluorescent, posing challenges to direct imaging. On the other hand, the lengthy decalcification process disrupted prolonged DSLS. These barriers hampered high-resolution imaging. Indeed, using regular approaches we could only detect very short DSLS-like structures in DTCs in the bone marrow (Supplementary Fig. 5a).

We experimented several procedures to overcome the above-mentioned difficulties. A bone-in-culture array (BICA)-based pipeline successfully revealed morphological features of cancer cells that colonize bones. Specifically, mouse hind limb bones containing either experimental bone lesions introduced via Intra-Iliac Artery Injection (IIA Inj.)³⁴ or spontaneous bone metastases were extracted and fragmented to generate ex vivo bone segments, which were then subjected to light microscopy and deconvolution microscopy in live tissues (Fig. 5a). This approach was previously utilized to screen for drugs that selectively target cancer–bone interactions³⁵. Using MCF-7 cells and IIA injection, we could detect



GFP-tagged microscopic bone lesions as well as cellular protrusions that are morphological similar to DSLS in cultures (Supplementary Fig. 5b, c). Cofilin knockdown appeared to decrease such protrusions in both MCF-7 (Supplementary Fig. 5d) and AT3 within the bone (Fig. 5b). Using the murine AT3 line, spontaneous bone metastases comprising a larger number of cancer cells also exhibited prolonged cellular protrusions that are likely to represent DSLS (Fig. 5c). To ask whether the suspected DSLS mediated interactions between cancer cells and osteogenic cells, we examined spontaneous bone metastasis derived from

RFP⁺ AT3 cells in osterix (OSX)-GFP mice in which osterix-expressing pre-osteoblast and osteoblast cells are GFP⁺. Under the BICA setting, we observed that cancer cells tend to localize to OSX-enriched regions and develop long cellular protrusions (Fig. 5c). In an independent experiment, GFP⁺ AT3 were injected with IIA into NG2-RFP mice. NG2 is a well-established marker of perivascular mesenchymal cells, and often used to indicate mesenchymal stem cells^{36,37}. Confocal imaging showed that AT3 DTCs develop cellular protrusions that often terminate on NG2⁺

Fig. 4 **Protrusions facilitate gap junctions, calcium transfer between the cells.** **a** MCF-7 E-cadherin (green) expressed where DSLS contacts osteoblast, here visible with phalloidin staining (red). Scale bars are 10 (left), and 5 μm (right), respectively. **b** MCF-7 E-cadherin (green) binding osteoblast N-cadherin (red) where DSLS contacts osteoblast. Scale bars are 5 (left), 1 μm (right), respectively. **c** Osteoblast gap junction protein connexin 43 (Cx43 – red) at the site of MCF-7 (green) DSLS contact. Scale bars are 5 (left), 1 (upper right), and 1 μm (lower right), respectively. **d** Gap junction permeable dye calcein red traversing from MCF-7 to osteoblast via DSLS. Scale bar is 15 μm . **e** Flow cytometry quantification of calcein dye transfer. The first panel shows un-transfected MCF-7 loaded with calcein red dye. The second shows GFP-labeled osteoblasts. The third shows the co-culture of the two cells after 18 h. **f** Box plots showing the 2D migration cells through the membrane per field for MCF-7 and AT3 cells treated with 10 μM CBX gap junction inhibitor with and without osteoblasts. Each dot represents a biological replicate, an average of three technical replicates. **g** Box plots showing the transwell migration cells through the membrane per field for MCF-7 cells treated with 10 μM CBX gap junction inhibitor with and without osteoblasts. Each dot represents a biological replicate, an average of three technical replicates. For box plots, the lower and upper hinges correspond to the first and third quartiles (the 25th and 75th percentiles). The upper whisker extends from the hinge to the largest value no further than $1.5 \times \text{IQR}$ from the hinge. The lower whisker extends from the hinge to the smallest value at most $1.5 \times \text{IQR}$ of the hinge. Data beyond the end of the whiskers are called “outlying” points and are plotted individually. *P*-value as determined by either ANOVA (**f**) or ANCOVA (**g**) as elaborated in “Methods”

mesenchymal stem cells, supporting the hypothesis that DTCs can form DSLS with osteogenic cells in the bone (Fig. 5d).

DISCUSSION

Our cell biology experiments strongly suggest that DSLS mediates MBT with osteogenic cells, and may drive migration of cancer cells that do not possess intrinsic migratory properties. This led us to hypothesize that physiological and pathological mobilization of osteogenic cells may provide momentum for DTC redistribution in the bone microenvironment. For example, bone fractures activate chemotaxis of MSCs toward the injured site and stimulate subsequent osteogenic differentiation³⁸. This process may coincide with migration of DTCs from the perivascular niche, where DTCs are kept dormant, to the osteogenic niche, where DTCs become proliferative. Consistent with this hypothesis, osteogenic MSCs have been reported to be perivascular in the resting stage^{37,39}, and therefore may provide a transition between the two different types of niches and an exit of the dormancy state. Significant future work will be needed to test this hypothesis and validate the functionality of DSLS in vivo. Given numerous gap junction-inhibitors and the discovery of cofilin-binding oligosaccharide JG6, itself shown to be capable of suppressing lung metastasis in mice, there may be a future translation of this data to the prevention or suppression of bone metastasis^{40,41}.

We realize that morphological evidence may not be sufficient to support the uniqueness of DSLS – it might indeed be molecularly related to one of these previously described structures. For instance, tunneling nanotubes (TNTs) have also been observed on MCF-7 cells in other studies^{42–44}. In fact, actin-based TNTs expressed by macrophages have been shown to contact breast cancer cells and confer upon them increased invasive properties, and TNT-deficient macrophages led to decreased xenograft tumor growth in vivo^{45,46}. However, there are significant differences in morphology between TNTs and DSLS in the same cell line (Supplementary Fig. 2b). In addition, at the functional level a cellular protrusion that mediates heterotypic cell-cell interaction and leads to co-migration between different cell types has not been described. Furthermore, the formation of such protrusions via initial contact and withdrawal, as well as the role of cofilin, is different than TNT formation described previously. In any case, it may be that DSLS and TNTs are closely related, but DSLS in context represent a new type of small actin-based protrusion function.

METHODS

Reagents and antibodies

General laboratory reagents and chemicals were obtained from Thermo Fisher Scientific or Sigma-Aldrich unless specifically indicated. Inhibitor stocks were prepared in water as follows: 50 mM carbenoxolone (CBX) (Sigma-Aldrich), 2 mg/mL Gap19 (Tocris Bioscience), 2 mM Latrunculin B

(Sigma-Aldrich), 100 nM Jasplakinolide (Sigma-Aldrich) and 250 nM Nocodazole (Sigma-Aldrich). Commercially available monoclonal antibodies (mAb) or polyclonal antibodies (pAb) were used for Cofilin (rabbit pAb, Abcam ab42824), GFP (chicken pAb, Abcam ab13970), E-cadherin (rabbit mAb, Cell Signaling Technologies 3195S), N-Cadherin (mouse mAb, Abcam ab98952), Notch3 (rabbit pAb, Abcam ab23426), Cx43 (rabbit pAb, Sigma C6219), Myosin X (rabbit pAb, Novus, 22430002) and ALP (rabbit mAb, Abcam ab108337). To stain for actin Texas Red™-X Phalloidin (Thermo-Fisher T7471) was used. Dye was diluted (1:40) in 100% ethanol and exposed to fixed cells for 20 min at room temperature. Secondary goat antibodies against chicken, mouse, or rabbit labeled with Alexa Fluor 488, 594 or Cy5 were purchased from Jackson ImmunoResearch Laboratories Inc.

Red CellTrace™ Calcein AM Red-Orange was used to track the formation of functional gap junctions between two cells. Dye was loaded by reconstituting 50 μg in 31 μL of anhydrous DMSO to make a 2 mM Calcein AM Stock Solution. Immediately prior to use, the Calcein AM Stock Solution was diluted in PBS to 2x Calcein AM Working Solution. Working solution was added to cells in suspension in PBS at a 1:1 ratio. The cells were then incubated for 30 min at 37 °C under 5% CO₂, then washed with PBS 4 times before seeding.

Molecular biology

pEGFP-N1 human cofilin WT was obtained from Addgene (Plasmid #50859) and cloned into pLJM1-EGFP (Plasmid #19319) by removing eGFP::Cofilin with Xba1 and Nhe1 and cloning into pLJM1-EGFP. EGFP was removed from pLJM1 with the same enzymes. Wild-type Cx43 and dominant negative Cx43 G138R was cloned from hFOB1.19 cDNA, mutations introduced by PCR. pInducer22 was used to express these genes in a doxycycline-induced overexpression system. To induce gene expression in vitro cells were treated with 50–100 ng/ml doxycycline in culture media.

GIPZ shRNA knockdown constructs were obtained from the BCM Cell-Based Assay Screening Core, and are originally from Dharmacon. They are as follows: GIPZ empty vector, Human Cofilin A V2LHS_64316, Human Cofilin B V2LHS_64314, Mouse Cofilin A V3LMM_449309, Mouse Cofilin B V3LMM_521443. Using Xtreme Gene HP DNA Transfection Reagent (Version 08, Roche), these vectors were transfected into 293T cells with pMD2.G (Addgene #12259) and psPAX2 (Addgene #12260) to package lentivirus. Lentiviral stocks were filtered by 0.45 μm polyethersulfone membranes (VWR 28145–505). Cancer cells were incubated with eGFP::cofilin lentivirus and 4 $\mu\text{g mL}^{-1}$ polybrene for 8 h. After a 72 h culture period, successfully labelled cells were isolated by puromycin selection and knockdown confirmation with qPCR and western blot. siRNA targeting cx43 was transfected using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher #13778). Cells were allowed to stabilize after transfection for 48 h, then seeded for migration assays. For western blots, the Electrophoresis and Membrane-Transfer were performed by iBlot (Invitrogen). Imaging was obtained by Odyssey (Li-Cor) system, following the manufacturer's protocol.

Cell culture procedures

MCF7, MDA-MD231, SCP28, 4T1, 4T1.2, and Raw 264.7 were cultured in DMEM with 10% (vol/vol) bovine calf serum and 1% pen strep amp at 37 °C and 5% CO₂. T47D ZR-75-1 were cultured in RPMI-1640 at 37 °C and 5% CO₂ (T47D media was supplemented with 0.2 Units/ml bovine insulin). hFOB 1.19, MSCs, MC3T3-E1 were cultured in phenol red-free DMEM/F12

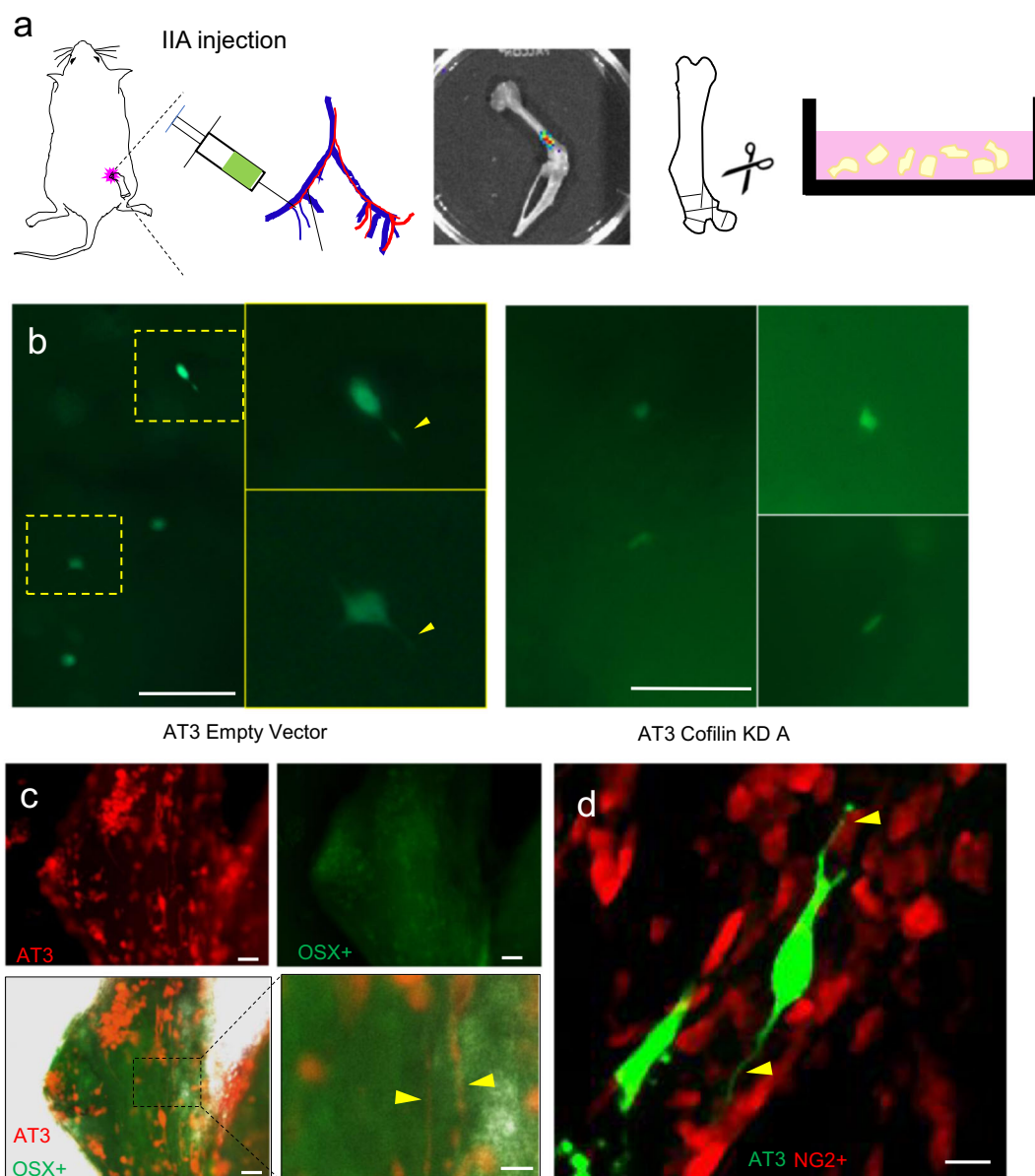


Fig. 5 Evidence of DSLS in breast cancer bone metastasis. Labeled AT3 cells were injected via intra-iliac artery (IIA) injection into C57B/6 mice. Mice were immediately sacrificed, hind limb bones harvested, and cut into small (1–2 mm diameter) pieces. Bone fragments are cultured over the next 3 days and imaged each day. **a** AT3 cells with both empty vector and cofilin knockdown (KD) shRNA transfection in the bone 24 h after intra-iliac artery injection, sacrifice, and bone culture. Cofilin knockdown decreased the proportion of DTCs with visible DSLS from 50% to 16.7% ($n = 9$ mice). Yellow arrows indicate potential DSLS. Scale bars are 150 μ m. **b** Spontaneous bone metastasis generated by orthotopic mammary tumors of RFP-tagged AT3. Hosts were OSX-GFP mouse mice. Red, green and merged channels are shown separately. Yellow arrows indicate potential DSLS in the vicinity of OSX+ cells. Scale bars are 50 (upper left), 50 (upper right), 50 (lower left), and 20 μ m (lower right). **c** NG2-RFP mouse bone fragment containing GFP-tagged AT3 cells delivered by intra-iliac artery injection. Yellow arrows indicate DSLS from RFP-tagged AT3 cells terminating in contact with NG2+ mesenchymal stem cells. Scale bar is 15 μ m.

a:1 with 10% (vol/vol) bovine calf serum and 1% pen strep amp at 34 °C and 5% CO₂. T47D ZR-75-1 at 37 °C and 5% CO₂.

Migration assays

All co-culture experiments were in phenol red-free DMEM/F12 1:1 with 0.2% FBS and 1% pen strep amp at 37 °C and 5% CO₂. Removable silicone culture-inserts were purchased from Ibidi-Fisher Cat. No: 80369. For 2D cell migration assays, 5000 cells per cell type were seeded into silicone chamber and allowed to attach for one hour before removal, and imaged over 48 h in a Sartorius IncuCyte S3 long term live imager at $\times 4$ magnification. Cells were counted in fields sized 1.25 mm by 1.00 mm immediately adjacent to removed silicone barrier (long edge parallel to removed barrier), chosen at random but fully flush to edge. Three fields per

well were averaged for a single replicate. The researcher was not blinded to treatment group during quantification. For transwell assays, 10,000 cells per cell type were seeded into the top chamber in 100 μ L of co-culture medium, and allowed to attach for one hour before the addition of 650 μ L chemoattractant/drug-containing co-culture media to the bottom well. 6.5 mm inserts with a pore size of 8 μ m were purchased from Corning (3422). Human Bone Morphogenetic Protein-2 (BMP-2) was purchased from Alfa Aesar™/Fisher (J67332EXE). Epidermal growth factor (EGF) Recombinant Human Protein was purchased from Thermo Fisher Scientific (PHG0311L). At 48 hours, cells were scraped from the top of the insert with a cotton swab, then the inserts were fixed in 5% glutaraldehyde for 10 minutes, and finally rinsed with water. Transwell cells were counted using fluorescence on a Leica DMI8 with a 20 $\times$ /NA objective. Three fields per insert were chosen at random and averaged to form a single replicate.

The researcher was not blinded to treatment group during quantification. For anti-proliferation control (Supplementary Fig. 1f) 2 μ M thymidine was added. For extracellular matrix control (Supplementary Fig. 1h), osteoblasts were incubated with the transwell inserts for 24 h before trypsin-free removal with EDTA, and the addition of cancer cells. Live imaging chemotaxis was captured using Incucyte ClearView plates and imaged every 2 h for a total of 70 h. Raw data available online⁴⁷.

Live-cell microscopy

Live cell imaging was performed with several different technologies. Time lapse imaging was achieved using an Incucyte S3 at 4x or 10x magnification at 37°C and 5% CO₂. Images were taken every hour for a minimum of 48 h and combined to form time-lapse videos. Biotek Cytation 5 Epi-fluorescence microscope time lapse imaging and GE Healthcare Deltavision DV Live imaging were done with the assistance of the Integrated Microscopy Core at Baylor College of Medicine. Both of these imagers utilized environmental control at 37°C and 5% CO₂. Image frequency varied by experiment. eGFP::cofilin live imaging was performed on a Nikon Eclipse TS100 with DS-Fi2 camera.

Immunofluorescence microscopy

Cells seeded onto Nunc™ Lab-Tek™ II CC2™ 4-well chambers slides were fixed with 4% paraformaldehyde for 10 min at 0°C and permeabilized using 100% ethanol at 0°C for 10 min. Samples were then blocked with 5% normal goat or donkey serum in 10% BSA PBST (PBS + 0.2% (vol/vol) Triton X-100). Primary antibody labeling was performed in 10% BSA PBST overnight at 4°C (1:1000), while secondary antibody (1:250) incubation was for 2 h at room temperature in the same solution. After DAPI stain these samples were mounted with ProLong gold antifade (Thermo Fisher Scientific, P36930). Imaging was performed on a GE Healthcare DeltaVision LIVE Deconvolution Microscope with the assistance of the Integrated Microscopy Core at Baylor College of Medicine. Imaging was performed using an Olympus UApO 40x/1.35 objective, z stacks were acquired at 0.4 μ m optical spacing covering the whole of the cells in field before applying a conservative restorative algorithm for quantitative image deconvolution in SoftWorx v7.0, 10 cycles were used for all the images. Max intensity projections were then generated and used in the figures.

For mouse bones immunolabeling, the harvested hind limb bones were fixed in 4% PFA overnight then decalcified in 14% EDTA over the course of a week. Bones were then embedded in paraffin and sectioned with assistance by the Baylor College of Medicine Breast Center pathology core. Sections were treated in a 55°C oven for 1–2 h, then incubated in xylene 3 times for five minutes each, before rehydration in 3 min incubations in 100%, 95%, 70%, 50% ethanol and finally water. Antigen retrieval was performed by 10 min, 95°C citric acid treatment. After PBS washes, slides were blocked in 10% serum in PBST. Primary treatment was incubation in primary antibody diluted with 1.5% serum (donkey and/or goat) in PBST overnight at 4°C. Secondary antibody treatment was 30 min (1:250 alexa 488, 594) diluted in PBST. Slides were treated in 1:1000 DAPI for 10 min and sealed under cover glass with anti-fade solution and nail polish.

Correlative light and electron microscopy and cryoET

QUANTIFOIL® Gold 200 mesh london finder grids with an R2/2 holey carbon film of round 2 μ m holes with 2 μ m spacing (Quantifoil Micro Tools GmbH) were placed in the 10 mm glass well of a MatTek 35 mm cell culture dish, then coated with collagen overnight. Red-labeled MCF-7 cells and GFP-labeled osteoblast pairs sorted by FACS were plated and grown on the grids overnight. Grids were then transferred to the blotting chamber of a Leica EMGP vitrification device at 37°C and 90% humidity. Inside the blotting chamber, 3 μ l of BSA-treated 15 nm gold fiducial marker solution (Electron Microscopy Sciences Cat# 25489) was added to the sample-side of the grid immediately prior to blotting from the back-side of the grid using Whatman 541 ashless filter paper (GE Healthcare part #1541055) and plunging into nitrogen-cooled liquid ethane at -168°C . Blot times of grids with successfully vitrified cells ranged from 3–5 s.

Tilt series were collected at 200 kV on a JEOL JEM2200FS energy-filtered field-emission cryo-electron microscope using a Gatan Model 895 UltraScan 4000 4k $\times$ 4k CCD camera and a Gatan Model 626 side-entry cryo-specimen holder (Gatan, Inc.). The nominal magnification used for imaging was $\times 15,000$ with a calibrated pixel size of 7.64 Å/pixel. Data collection was performed using SerialEM⁴⁸ with a target defocus of $-6 \mu\text{m}$, a tilt range of -60° to $+60^\circ$ 3 degree increments, and a total electron dose of ~ 60 electrons/Å². Fiducial-marker-based tilt-series alignment and 3-

dimensional tomographic reconstruction by back-projection were performed using IMOD⁴⁹. Annotation of cellular features was performed manually using Amira (Thermo Fisher Scientific).

Flow cytometry

Flow cytometry was performed as previously described. Calcein Red loaded MCF-7 cells and GFP-labeled osteoblasts were co-cultured for 18 h before being captured by trypsin, washed, and analyzed with BD FACS Aria II with help from the Baylor College of Medicine Cytometry and Cell Sorting Core.

Animals

C57B/6, NRG, athymic nude mice, Sp7-tTA,tetO-EGFP/cre (OSX-GFP) mice, B6.Cg-Tg(Cspg4-cre;Esr1*)BAkik/J (NG2-CreER), and B6.Cg-Gt(ROSA)^{26Sortm14(CAG-tdTomato)Hze/J} (Ai14D) mice were purchased from Jackson Laboratory. NG2-RFP mice were generated by crossing NG2-CreER and Ai14D mice. The strain and number of mice used for each experiment are mentioned in text and legends. All animal work was done in accordance with a protocol approved by the Baylor College of Medicine Institutional Animal Care and Use Committee. The investigator was not blinded to the group allocation during the whole experiment.

Ex vivo bone imaging

Intra-iliac injections and mammary gland injections were performed as previously described. Mice were sacrificed 24 h after IIA injection and the femur and tibia were harvested, cut into 1–2 mm fragments and cultured in DMEM 10% FBS media, imaged daily. For spontaneous metastasis, mammary gland injected tumors were allowed to grow to 1 cm diameter, then resected. Mice were sacrificed after about 18 days before bones were harvested, cut into fragments, and cultured. Bone fragments were imaged on both Echo Revolve R4 and GE Healthcare Deltavision DV.

Statistics

We used analysis of variations (ANOVA) and analysis of covariance (ANCOVA) to compute statistical significance based on data generated by independent experiments. In both cases, normalized numbers of cell migration (cell counts over area of the field) were used as response. In BMP-2-chemotaxis transwell migration assays, the covariates include BMP-2 concentrations as a continuous variable. Other variables, including the presence of OBs, knockdown of cofilin, and treatment of CBX are categorical. Importantly, independent experiments were also included as categorical variables to control for inter-experimental variations. A generalized linear model was then fit using the “lm” package of R. The reported *p*-values were computed between the experimental groups indicated by the straight lines in each figure with covariate of independent experiments. For DSLS+ proportion, error calculated by binomial distribution.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

DATA AVAILABILITY

The data generated and analysed during this study are described in the following data record: <https://doi.org/10.6084/m9.figshare.12682523>⁴⁷. This data record contains all data and supplementary data referred to from this paper.

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AUTHOR CONTRIBUTIONS

A.M.M., W.Z., and L.J. performed cell culture and in vivo experiments, W.D. and P.G.M. did CryoEM imaging and analysis, F.S. and M.A.M. assisted in imaging and image analysis, H.W. W.C. and X.H-F.Z. provided guidance and scientific input. A.M.M. and X.H-F.Z. wrote the paper.

COMPETING INTERESTS

The authors declare no competing interests.

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Exploiting bone niches: progression of disseminated tumor cells to metastasis

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Many solid cancers metastasize to the bone and bone marrow (BM). This process may occur even before the diagnosis of primary tumors, as evidenced by the discovery of disseminated tumor cells (DTCs) in patients without occult malignancies. The cellular fates and metastatic progression of DTCs are determined by complicated interactions between cancer cells and BM niches. (2. AUTHOR: "BMN" appeared only a few times and was more often spelled out. For consistency, we have spelled it out as "BM niche" throughout. Is this OK?) Not surprisingly, these niches also play important roles in normal biology, including homeostasis and turnover of skeletal and hematopoiesis systems. In this Review, we summarize recent findings on functions of BM niches in bone metastasis (BoMet), particularly during the early stage of colonization. In light of the rich knowledge of hematopoiesis and osteogenesis, we highlight how DTCs may progress into overt BoMet by taking advantage of niche cells and their activities in tissue turnover, especially those related to immunomodulation and bone repair.

Introduction

The bone is a common site of metastasis for many tumor types, particularly those of breast, prostate, lung, and multiple myeloma (1–3). Breast and prostate cancers are the most common types of solid tumors in women and men, respectively. As many solid tumors can be surgically removed, metastasis is the real risk to these patients, causing over 90% of cancer-related deaths (4, 5). However, effective therapies for metastases, including bone metastases, are still lacking (6).

Metastases and primary tumors often occur asynchronously. The interval between primary tumor removal and diagnosis of metastases is referred to as metastasis dormancy (7, 8). During metastasis dormancy, microscopic metastases or single disseminated tumor cells (DTCs) survive adjuvant therapies. A fraction of them finally outgrow and become overt metastases (9, 10). A significant proportion of patients have DTCs detected in the bone marrow (BM) at the time of diagnosis (11–13), and even after tumor resection (14, 15), indicating that distribution of these seeds to different organs occurs earlier than diagnosis (16, 17), and therefore may be inevitable with current detection limits. However, targeting the ability of DTCs and micrometastases to adapt to and colonize secondary sites may represent a viable approach to eliminate metastases (18).

Metastatic cancer cells that make it to the bloodstream are subject to immune attack, physical shearing, and other forces

(19–21). However, some of these cells are able to travel around the body and extravasate through the endothelial wall into the tissue (22). The BM is a particularly accessible and nurturing nest for DTCs, as sinusoidal capillary beds with fenestrated endothelium are permissive for cancer cell extravasation (23). The BM microenvironment, as the cradle of hematopoiesis, evolved to enrich growth factors (e.g., IGFs and FGFs) and cytokines that normally support stem cell activities of both hematopoietic and mesenchymal lineages (24). Therefore, it is not surprising that the BM niches of DTCs resemble those of hematopoietic and mesenchymal stem cells. Indeed, cancer cells may hijack unique homeostatic and regenerative functions of these niches to facilitate their own survival and progression. In this article, we review recent findings on the roles of BM niches in metastatic colonization. We focus on how cancer cells exploit activities of the niche components to promote metastatic colonization. These activities may be related to their normal functions such as establishing an immune-privileged environment, maintaining tissue homeostasis, and healing wounds.

Bone homeostasis

Bone is a remarkable organ that plays complex physiological roles, such as providing structural support and facilitating hematopoiesis (25–27). The unique bones of the body are diverse in size and shape, with differing amounts of cortical bone (compact and dense) as well as trabecular (or spongy) bone. Bone is maintained by cells of the osteoblast and osteoclast lineages (28, 29). Mesenchymal stem cells develop into preosteoblasts, then mature osteoblasts (30, 31). These cells produce both the mineralized portion of bone, hydroxylapatite, and organic components: collagen, osteocalcin, and osteopontin (32). Mature osteoblasts line the mineral

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1 surface within the bone (33). The osteoid, or organic, components
2 are deposited first; as the tissue becomes mineralized and new
3 bone is formed, osteoblasts become trapped within and further
4 transform into osteocytes (34, 35). The osteocytes form long mem-
5 brane protrusions that connect to each other through thin chan-
6 nels called canaliculi (36), communicating through gap junction
7 channels at their tips (37). Osteoclasts, responsible for breaking
8 down the mineralized bone tissue, develop from the monocyte/
9 macrophage lineage upon stimulation by NF- κ B/RANKL as well
10 as macrophage colony-stimulating factor (38, 39). Osteoclasts are
11 large, multinucleated cells that become strongly polarized toward
12 the bone surface for resorption, and their ruffled cell edges attach
13 to bone (40). Bone resorption is accomplished by secretion of
14 proteases to dismantle organic bone components and protons (3.
15 **AUTHOR: Is “proteins” intended here?)** to break down inor-
16 ganic mineral (41).

17 Hematopoietic stem cells and their BM niches

19 Hematopoietic stem cells (HSCs) originate during embryogenesis
20 (42, 43), expand in fetal tissues (44), and ultimately home to the
21 BM (45), where they maintain lifelong hematopoiesis. Instead of
22 randomly distributing in the BM, HSCs reside in specialized BM
23 niches. Two types of BM niches harbor HSCs and tightly regu-
24 late their fates: the perivascular niche and the endosteal niche
25 (46). The perivascular niche is subdivided into a sinusoidal and
26 an arteriolar portion. The majority of SLAM family markers that
27 label HSCs (Lin⁻, CD48⁻, CD41⁻, CD150⁺) are localized in the
28 sinusoidal perivascular niche (47, 48), which comprises endothe-
29 lial cells and adjacent perivascular stromal cells, including leptin
30 receptor-expressing (LEPR-expressing) cells (49, 50), nestin-ex-
31 pressing cells (50, 51), and CXCL12-abundant reticular (CAR) (4.
32 **AUTHOR: “CAR” does not appear elsewhere; can the abbrevi-**
33 **ation be omitted here?)** cells (52). On the other hand, a small
34 fraction of HSCs are found adjacent to the arteriolar perivascular
35 niche (53, 54), where they come into close contact with LEPR⁺ peri-
36 vascular cells, non-myelinating Schwann cells (55), and NG2-ex-
37 pressing stromal cells. These perivascular cells secrete important
38 cytokines such as CXCL12 (also known as SDF-1), stem cell factor
39 (SCF), and TGF- β (55), which make distinct contributions to HSC
40 maintenance (50). Although active HSCs are localized to the peri-
41 vascular niche, some studies showed that quiescent HSCs mainly
42 reside in the endosteal niche with arterioles (47, 56–58), which is
43 composed mainly of osteogenic cells. HSCs may depart from one
44 niche and enter another niche in response to specific signals (53,
45 59, 60). Interestingly, some studies suggested that HSCs prefer
46 to engraft at the endosteal region after transplantation (61, 62).
47 Although the perivascular niche and the endosteal niche are cel-
48 lularly distinct niches and may harbor distinct subpopulations of
49 HSCs, a part of these two niches may (5. **AUTHOR: Is a word**
50 **missing here?)** spatially close to each other in the endosteum
51 region, since blood vessels are also rich near the bone. However,
52 a cross section of the tibia shows that HSCs are modestly enriched
53 in the central BM and reduced toward the endosteum in the diaph-
54 ysis (48). Interestingly, evidence reveals that type H endosteal ves-
55 sels can regulate osteoprogenitors, thereby coupling angiogenesis
56 to bone development, indicating complex crosstalk among BM
57 niche cells (63, 64).

In addition to the endosteal niche and perivascular niche, 1
other studies also revealed that megakaryocytes, a terminally 2
differentiated hematopoietic cell, serve as a niche component 3
that directly or indirectly regulates HSC fate (65–68). Megakary- 4
ocytes predominantly occupy the sinusoidal perivascular niche 5
(6. **AUTHOR: Changed “are predominantly occupied in” to** 6
“predominantly occupy”. Is your meaning as intended?) and 7
contribute to maintaining HSC quiescence by producing CXCL4 8
and TGF- β (69). 9

The cellular fates of HSCs, namely dormancy, self-renewal, 10
and differentiation, are regulated by microenvironmental signals 11
emitted from specific niches. During homeostasis, niche signals 12
keep a proportion of HSCs dormant to preserve their long-term 13
self-renewal capacity (57, 58, 70), while other homeostatic HSCs 14
are activated to drive normal hematopoiesis. The current model 15
of hematopoiesis states that the differentiation of HSCs follows 16
a strict hierarchy (71). Long-term reconstituting HSCs possess 17
multilineage differentiation potential and differentiate into short- 18
term reconstituting HSCs (ST-HSCs), which exhibit more-limited 19
self-renewal potential. ST-HSCs further generate multipotent 20
progenitors (MPPs) with distinct differentiation potential. These 21
MPPs rapidly divide into oligopotent progenitors, including com- 22
mon lymphoid progenitors (CLPs) and common myeloid progeni- 23
tors (CMPs). CMPs then produce granulocyte-monocyte progeni- 24
tors (GMPs), megakaryocyte-erythrocyte progenitors (MEPs), and 25
dendritic cell (DC) progenitors. These oligopotent progenitors 26
advance to their lineage-restricted precursors and ultimately give 27
rise to all terminally differentiated blood cells. Specifically, MEPs 28
differentiate into erythrocytes and platelet-producing megakary- 29
ocytes. GMPs generate neutrophils, basophils, eosinophils, and 30
monocytes (which can further differentiate into macrophages in 31
the BM and peripheral tissues). CLPs are precursors of DCs, B 32
cells, T cells, and NK cells. However, HSCs may also be hetero- 33
geneous and can be organized into a cellular hierarchy and exist 34
(7. **AUTHOR: (a) A word(s) seems to be missing here. Please** 35
clarify. (b) Do all edits to this sentence express the correct 36
meaning?) distinct cell differentiation routes, some of which 37
may skip intermediate steps. For instance, the platelet-producing 38
megakaryocytes can skip the intermediate oligopotent progenitors 39
and directly generate from megakaryocyte-primed HSCs (72, 73). 40

Taken together, all the above-mentioned skeletal and hema- 41
topoietic cells constitute an intricate environment for metastatic 42
cancer cells. It should be noted that these cells are not static but 43
are rather highly dynamic in proliferation, differentiation, and 44
relocation. How these dynamics impact seeding and colonization 45
of DTCs remains elusive. 46

47 BM niches in metastasis

48 With the above in mind, we will review the roles of various 49
bone-resident cells throughout the metastatic colonization pro- 50
cess. Since a large proportion of literature in this area is based on 51
breast cancer (BCa) models, we will focus on this cancer type, and 52
summarize prominent features of other cancer types in separate 53
sections. 54

The pre-metastatic niches. The impact of BCa on the BM niches 55
starts even before DTCs arrive in the bone, a notion known as the 56
“pre-metastatic niche.” There, BM-derived hematopoietic cells 57

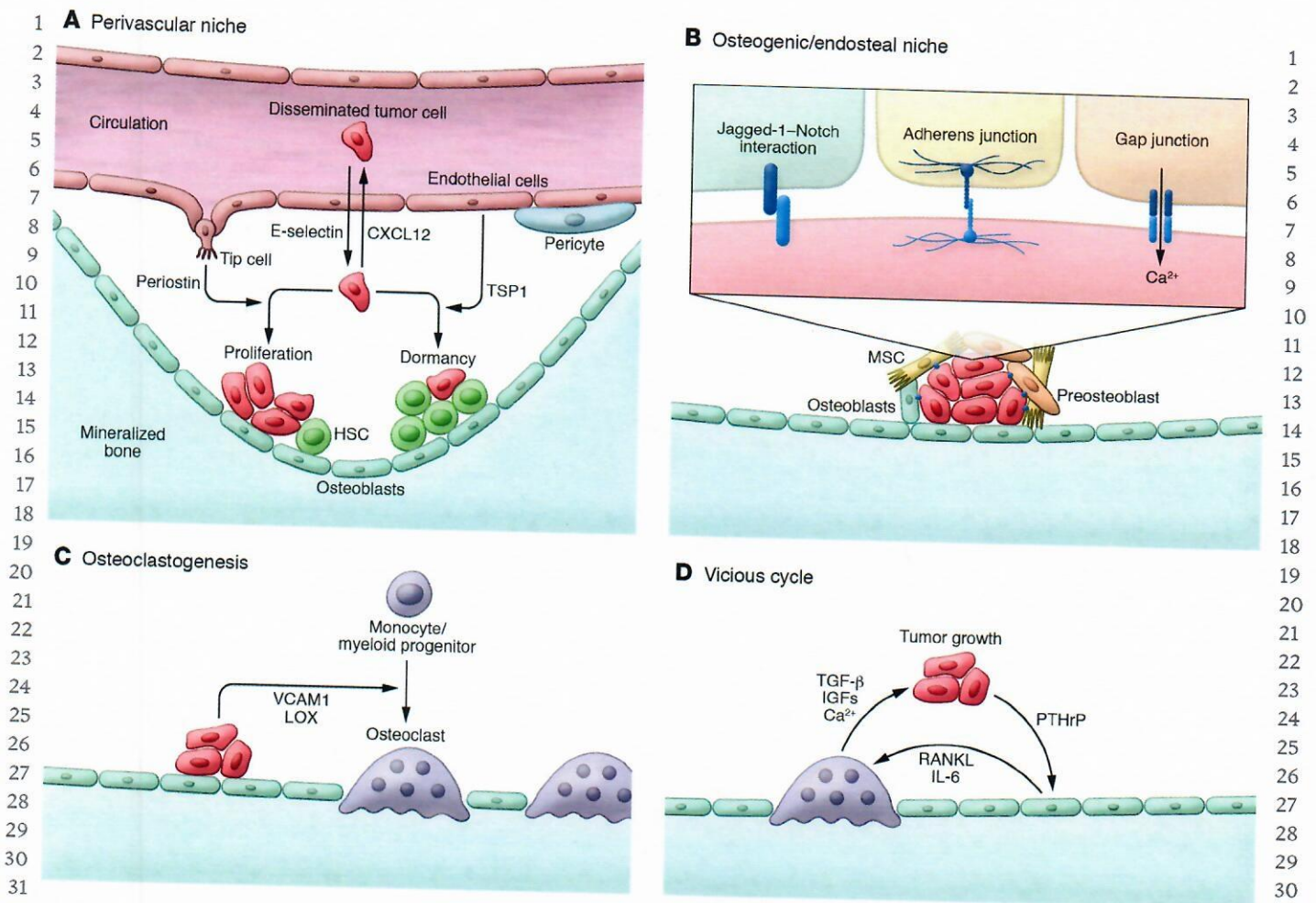


Figure 1. Disseminated tumor cells utilize bone marrow niches to survive and colonize. Disseminated tumor cells (DTCs) occupy many niches in the bone after they arrive in the organ via the bloodstream. When cells first extravasate from the vessels, they may reside in the perivascular niche. DTCs occupy and compete with the HSC niche through recruitment by CXCR4/CXCL12 signaling to the perivascular niche. Several signaling pathways and molecules govern the dormancy/proliferation fate of cancer cells in this niche, including pericyte-derived thrombospondin-1 (TSP1). Cancer cells can also interact with cells of the osteoblast lineage, namely mesenchymal stem cells, preosteoblasts, and osteoblasts, which constitute the osteogenic niche. Here they use heterotypic cellular junctions as well as Jagged-1-mediated Notch signaling to crosstalk with the niche. Gap junctions formed between the cancer cells and osteogenic cells facilitate calcium transfer into the cancer cells. These interactions collectively promote DTC proliferation and chemoresistance. Osteoclastogenesis may be activated by multiple mechanisms, which leads to a feed-forward loop known as the vicious cycle. In this cycle, tumor cells produce both pro-osteoblastic and pro-osteolytic factors, stimulating both osteoblasts and osteoclast activity. The destruction of the bone releases embedded growth factors and parathyroid hormone-related protein. (21. AUTHOR: JCI staff adjusts figure size and edits labels to conform to JCI style and format. Please check all figures and confirm that all labels are correct.)

were shown to “prepare the soil” for subsequent metastatic seeding, and subsequent studies supported this finding (74), including some suggesting that the pre-metastatic niche’s impact on metastasis can be bidirectional (75, 76). Although BM-derived cells play major roles in modeling the pre-metastatic niche in other organs, the effects of primary tumors on BM itself were not studied until recently. Cox et al. showed that xenograft mammary tumors secrete lysyl oxidase (LOX), which generates systemic effects and activates osteoclastogenesis, thereby priming the formation of osteolytic lesions once cancer cells have seeded (77). Hoshino et al. reported that the metastatic site can receive exosomes derived from primary tumors, which facilitate subsequent colonization in an organ-specific fashion (78). Although bone-tropic exosomes were not identified in this study, lung-tropic exosomes divert-

ed metastatic seeding of bone-targeting cancer cells from bone to lungs, supporting the notion that exosomes help determine organotropism of metastasis through effects on the pre-metastatic niche. Lung cancers also crosstalk with bone before metastasis occurs. While the presence of tumors increases bone mass, osteoblasts return the favor by supplying a specific subset of neutrophils that promote tumor progression (79). Taken together, it has become increasingly clear that BM niches already begin to change under the influence of primary tumors before metastasis occurs. Further studies are needed to characterize these changes thoroughly to determine their functional impact on subsequent metastatic seeding and colonization.

The perivascular niche. When DTCs arrive via the bloodstream to the bone, they extravasate by interacting with the endothelium,

1 which is particularly permeable in the BM (80) (Figure 1). Here, the
 2 cells reside in the perivascular niche (81, 82), which harbors HSCs
 3 as described above. This location also contains various immune
 4 cells and pericytes that possess activities of mesenchymal stem
 5 cells (MSCs) (83–85). There is some debate as to whether MSCs
 6 and pericytes are distinct cell types (86); nevertheless, these endo-
 7 thelial-adjacent cells have significant impact on DTCs when they
 8 arrive. Multiple signaling pathways may mediate the interactions
 9 of cancer cells with the perivascular niche and determine their fate
 10 accordingly. For example, the balance between the adhesion mol-
 11 ecule E-selectin and CXCL12 may be critical for DTCs to remain
 12 in the perivascular niche (82), whereas the counteractions (8.
 13 **AUTHOR: Is “Counteractions” as intended?**) between throm-
 14 bospondin-1 (TSP1) and TGF- β /periostin determine quiescence
 15 versus proliferation fate (81). Furthermore, vascular E-selectin
 16 may also trigger mesenchymal-to-epithelial transition and the
 17 acquisition of stemness traits, thereby fueling further progression
 18 (87). Interactions with the perivascular niche also render cancer
 19 cells resistant to chemotherapies (88). Overall, it is intriguing to
 20 note that the perivascular niche is a common site for DTCs and
 21 HSCs, both of which may alternate between quiescence and pro-
 22 liferation states. Interestingly, the perivascular region has the
 23 highest degree of hypoxia in the bone, an already hypoxic organ
 24 in general (89). Hypoxia plays both direct and indirect roles in
 25 the promotion of metastatic progression and is expounded upon
 26 in further reading (90, 91). Given the BM’s complicated and het-
 27 erogeneous vascular network, further work will be needed to com-
 28 pare the exact DTC and HSC distribution and the molecular com-
 29 positions in the corresponding niches.

30 *The osteogenic/endosteal niche.* The endosteal niche is located,
 31 as the name suggests, adjacent to the endosteum, or the lining
 32 of the BM cavity in adult bone. This niche is composed of osteo-
 33 clasts, osteoblasts (and their progenitors), and fibroblasts in multi-
 34 ple stages of development (92) (Figure 1). As described above, the
 35 endosteal niche also plays an important role in harboring and sus-
 36 taining HSCs. HSC-DTC competition for the endosteal niche was
 37 first reported in 2011 (93), thereby connecting the distinct fields
 38 of metastasis and hematopoiesis. Subsequently, an overlapping
 39 osteogenic niche has been described that promotes proliferation
 40 and early-stage bone colonization of BCa DTCs (94–96). In the
 41 osteogenic niche, luminal BCa DTCs form heterotypic adherens
 42 junctions with MSCs and preosteoblast and osteoblast cells, with
 43 E-cadherin on the cancer cell side and N-cadherin on the mesen-
 44 chymal cell side (96). In fact, as the niche develops and the lesion
 45 progresses, the DTCs become completely surrounded by the
 46 osteoblast-lineage cells. These junctions facilitate Notch signal-
 47 ing, which in turn renders metastasis resistant to chemotherapies
 48 (97). The junctions also lead to the formation of functional gap
 49 junctions between the cells through which cancer cells receive a
 50 calcium flux from osteogenic cells (95). The tumor cells are ineffi-
 51 cient at absorbing calcium from the matrix and therefore depend
 52 on osteogenic cells in the niche (95). The DTCs receive a growth
 53 advantage in the form of mTOR signaling activation in this niche
 54 and are able to progress from single cells to multicellular lesions
 55 (96). Perturbation of cadherins, gap junctions, or mTOR signaling
 56 decreases luminal cell colonization. Importantly, bone histomor-
 57 phometry revealed active osteogenesis within the niche (96), sug-

gesting ongoing tissue hemostasis.

The perivascular and osteogenic niches are not complete-
 ly separated. Some niche components are linked by lineage. For
 instance, a subset of pericytes have MSC activities and can dif-
 ferentiate into osteoprogenitor cells (83), raising the possibility
 of conversion from the perivascular niche into the osteogenic
 niche. In fact, a specific subtype of vessels has been shown to cou-
 ple angiogenesis and osteogenesis in the BM (63). These vessels,
 termed type H, express endomucin and CD31, often localize to
 the growth plate and endosteum, and are closely associated with
 osteogenic cells and promote their progression via Noggin secre-
 tion (9. **AUTHOR: Is this sentence correct as revised for clar-
 ity?**) (63, 64). Interestingly, in prostate cancer, endothelial cells
 can transdifferentiate into osteoblasts, thereby facilitating the
 formation of osteoblastic lesions (98). Bone turnover and remodel-
 ing may change the dynamics of both niches and strengthen
 their crosstalk. Therefore, it is possible that DTCs may affect their
 neighborhoods and alter their cellular fates accordingly. This will
 be elaborated in a subsequent section.

The vicious cycle. While DTCs and bone micrometastases
 predominantly interact with perivascular and osteogenic niches
 where the roles of osteoclasts are limited, the advanced pathogen-
 ic bone metastases are often characterized by a feed-forward loop
 of bone cells and osteoclasts. This feed-forward loop has been
 described as the “vicious cycle” (3, 99–101) (Figure 1). Tumor cells
 can secrete parathyroid hormone-related protein (PTHrP) (10.
**AUTHOR: Changed “peptide” to “protein” as appeared in
 this term below; and abbreviated the term below for con-
 sistency. OK as done?**) to stimulate osteoblasts, which in turn
 produce osteolytic factors such as IL-11 and RANKL and promote
 osteoclast proliferation and activation (102, 103). The resultant
 increase in osteolytic activity causes lesions to form in the miner-
 alized bone tissue, releasing factors that have been sequestered in
 the bone, including tumor cell-stimulating growth factors such as
 IGFs and TGF- β (104–107), thereby fueling the feed-forward loop.

Recent studies uncovered additional molecular players
 involved in the feed-forward loop. Jagged-1, a TGF- β target gene in
 cancer cells, activates Notch signaling in osteoblasts and enhances
 the expression of IL-6, which consequently stimulates osteoclas-
 togenesis (11. **AUTHOR: Is this sentence correct as revised
 for clarity?**) (105). IL-6 can also be generated during senescence
 of osteoblasts (108). Integrin β_3 represents another TGF- β target
 gene that is specifically induced in the bone microenvironment
 and confers proliferative advantages on cancer cells (109). Can-
 cer cells and osteoclasts sometimes depend on the same signaling
 pathways, such as the RON kinase (110). Taken together, these
 new findings provide additional therapeutic targets for treatment
 of advanced osteolytic metastases.

Little is known about the transition from the early niches pre-
 dominantly composed of endothelial and osteogenic cells to the
 osteoclast-driven vicious cycle that is a hallmark of advanced bone
 metastases. One possible mechanism is that the development of
 the osteogenic niche may alter the secretome of both cancer cells
 and osteoblasts. Indeed, it was shown that some dormant cancer
 cells in the BM may recruit osteoclast progenitors by secreting
 a soluble form of VCAM1, of which integrin $\alpha_4\beta_1$ is the cognate
 receptor (111) (Figure 1).

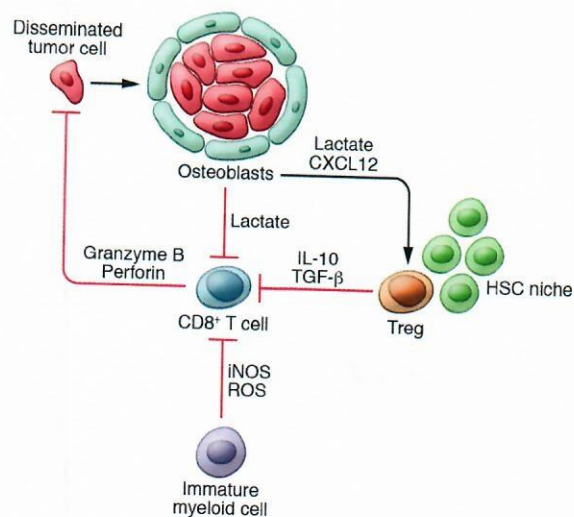


Figure 2. The immunosuppressive microenvironment in the bone and bone marrow. DTCs residing (22. **AUTHOR: Changed "reside" to "residing". Is your meaning as intended?**) in the bone marrow niches may produce many immunosuppressive factors that also interfere with cytotoxic CD8⁺ T cells. Among these factors are regulatory T cells (Tregs), immature myeloid cells, and osteoblasts. DTCs occupy and compete with the HSC niche to occupy the immune-privileged environment maintained by Tregs. Tregs secrete immunosuppressive cytokines (IL-10, TGF- β) and traffic to the HSC niche via the chemokine CXCL12. Immature myeloid cells develop in the bone marrow and generate immunosuppressive molecules (iNOS, ROS). Osteoblasts are a major source of the chemokine CXCL12 that attract Tregs and produce lactate as a metabolic by-product. Lactate is utilized by cancer cells as an intermediate in the production of ATP. Lactate has also been shown to inhibit cytotoxic CD8⁺ T cells, while Tregs maintain immunosuppressive function (23. **AUTHOR: Sentence correct as revised for clarity?**). Taken together, these factors within the bone marrow niches maintain an immune-privileged environment for DTCs to colonize. (24. **AUTHOR: Changed "GrB" to "Granzyme B" in the figure. Is this correct?**)

Prostate cancer. Prostate cancer bone metastases also activate a vicious cycle similar to that seen in BCa that can be both osteoblastic and osteolytic (112, 113). However, these metastases tend to be more osteoblastic in nature, as they express high levels of alkaline phosphatase (ALP) and osteocalcin (114). The increased bone formation is poorly regulated and dysfunctional, often leading to very dense lesions and rigid zones of bone with decreased overall bone strength (115, 116). Endothelin-1, a stimulator of osteoblasts, is overexpressed by prostate cancer cells, and thereby increases osteogenesis, ALP expression, and osteoblastic bone metastases (115, 117, 118). (12. **AUTHOR: The original ref. 117 was a duplicate of ref. 115. References have been renumbered accordingly throughout; please verify accuracy of renumbering.**) PTHrP produced by the tumor stimulates osteoblasts to produce the osteoclastogenesis-promoting factors MCP-1 and RANKL (119). Paradoxically, PTHrP-expressing tumor foci have areas of both increased osteoblastogenesis and osteoclastogenesis adjacent to them in the bone, indicating a more complex role for PTHrP (120, 121). Prostate-specific antigen can also tip the balance toward osteoblastic lesions by cleaving PTHrP (119, 121). To avoid detection and modulate bone homeostasis, prostate cancer cells may use osteomimicry, in which cells undergo (13. **AUTHOR: Is**

this sentence correct as revised for clarity?) osteoblastic-like differentiation, including expression of the osteogenesis markers BMP-2, RUNX2, and others, even to the point of actual mineral deposition (122).

Multiple myeloma and other cancers. Multiple myeloma, a cancer of plasma cells in the BM, can also cause lesions to form in the bone itself (123, 124). Patients can develop myeloma bone disease, when bony lesions cause fractures, pain, and other defects (125). In myeloma bone disease, cancer cells secrete osteoclastogenesis-promoting factors, including RANKL, IL-3, and IL-6 (126, 127), and also interact with BM stromal cells to produce such factors (128). This increased level of osteoclastogenesis is coupled with decreased or halted osteoblastogenesis, which is stimulated by IL-7, secreted frizzled-related protein, and other factors (125, 129). This results in characteristic lytic lesions in the bone that cause pain and fractures, and eventually patients can develop even more severe outcomes such as renal failure and anemia (130). More rarely, kidney, lung, skin, and thyroid cancers can also form osteolytic bone metastases (131).

Are the bone metastasis niches immune-privileged?

An often overlooked fact in bone metastasis (BoMet) research is that the bone is a major immunological organ. The hematopoietic niche within the bone is responsible for the production of erythrocytes and leukocytes, which requires a special environment that reduces aberrant immune activation. It was previously shown that the IRF7 pathway could facilitate escape of BoMet from immunosurveillance (132), which represents one of the earliest findings on the immunosuppressive milieu in BoMet. Given the complexity of BM niches, there might be multiple profound mechanisms contributing to the unique immune microenvironment. In this section, we summarize three likely cell types/pathways that may be responsible for immunosuppression: regulatory T cells, immature myeloid cells, and osteoblasts. Together, these mechanisms provide directions for further investigations into the evolving interactions between BoMet and the BM niches from the perspectives of tumor immunology and immunotherapies.

Regulatory T cells. As previously described, hematopoiesis occurs in the BM, leading to the formation of erythrocytes and leukocytes. Leukocytes can be further divided into two populations: myeloid and lymphoid cells. Among leukocytes, T cells are cells that mature in the thymus. Although maturation occurs in the thymus, mature T cell subsets can be found in the bone. A large portion of bone CD4⁺ T cells are regulatory T cells (Tregs) that contribute to immune suppression (133). FoxP3⁺ Tregs suppress the activity of proinflammatory cells, such as CD8⁺ T cells and Th1 CD4⁺ T cells. This immunosuppression occurs via Treg secretion of antiinflammatory cytokines such as IL-10, IL-35, and TGF- β (134, 135) (Figure 2). These cytokines may play a dichotomous role in promoting dormancy of metastatic seeds and increasing tumor cell proliferation.

The reduction of proinflammatory signaling near the perivascular niche may offer opportunity for dormant cancer cells to evade immune surveillance. As previously stated, bone is a privileged immunological organ that benefits from a reduction in immune activity; therefore, the further reduction of an inherently low baseline of immune activity may create a favorable location for dormant cell immune evasion (136). Additionally, TGF- β pro-

duction increases the proliferation of metastatic seeds and further perpetuates the vicious cycle of macrometastasis (102, 104). Tregs within the bone have also been shown to affect metastasis through attraction by chemokines. Previous studies showed the importance of CXCL12/CXCR4 signaling both in trafficking of Tregs to the bone and in the attracting of metastatic seeds in prostate cancer and BCa (14. **AUTHOR: Is this sentence correct as revised?**) (93, 137). Patients with prostate cancer displayed an increase in the number of Tregs within the bone through an increase in the expansion of Tregs by RANK signaling on DCs (138).

Metabolic effects may also enhance Treg function in the bone microenvironment. Micrometastatic lesions interact with osteoblasts that are found in the endosteum where Tregs localize and may lead to an increase in proliferation to catalyze the formation of the vicious cycle (96). These osteoblasts, as will be discussed in a subsequent section, prefer aerobic glycolysis that results in the accumulation of lactate, thereby creating an environment suitable for Treg function while inhibiting cytolytic T cell function. The presence of Tregs in the HSC niche and adjacent regions of the bone may provide metastatic seeds with an evasive environment for dormancy and proliferation.

Immature myeloid cells. Along with the production of lymphocytes, HSCs give rise to myeloid cell populations including neutrophils, monocytes, and macrophages. However, during development, a group of myeloid cells remains immature and can exert immunosuppressive function. Therefore, this subset of immature myeloid cells is often referred to as myeloid-derived suppressor cells (MDSCs). Immature myeloid cells (IMCs) are among the most abundant immune cell types found within the bone, because they are directly produced within the HSC niche. IMCs are a heterogeneous population of cells containing polymorphonuclear (PMN) and monocytic (M) MDSCs based on their morphology. Functionally, PMN-MDSCs contain higher levels of reactive oxygen species (ROS), while M-MDSCs contain higher levels of nitric oxide (NO) and inducible NO synthase (iNOS/NOS2) (139) (Figure 2). Previous studies have shown that there is an increase in the production of IMCs, including MDSCs, in cancer patients (140). This increase in abundance of immunosuppressive IMCs could further promote metastatic seeding, similar to the effects of Tregs. MDSCs have also been shown to produce TSP1, a factor that promotes dormancy, which may aid the early DTCs in remaining undetected in an immunosuppressive environment (75). The overlap of the metastatic niche with the location of IMC production may allow micrometastatic lesions to skew myeloid cell development down an immunosuppressive path. As explained by the model of immunoediting, cancer cells must evade the initial immune surveillance to progress to a state of immune evasion by recruiting immunosuppressive cells and promoting their development (141). Therefore, outgrowth of these micrometastatic lesions may lead to secretion of many antiinflammatory cytokines along with factors to skew development of HSCs toward myelopoiesis.

Osteoblast metabolism. The endosteal region of the bone undergoes constant remodeling as bone resorption and bone formation occur on the endosteal surface. Osteoblasts are responsible for bone formation and develop from MSCs/osteoprogenitors that differentiate into preosteoblasts (31), all of which play a major role in early-stage bone colonization as previously described in

The osteogenic/endosteal niche section above.

The influence of osteoblasts during early-stage metastasis may also involve suppression of the immune system due to aerobic glycolysis, the preferred form of osteoblast metabolism. Under conditions of aerobic glycolysis, osteoblasts convert glucose into lactate in the presence of oxygen (142). This lactate-rich environment has been shown to be beneficial to metastatic seeds, and the inhibition of lactate dehydrogenase (LDH) reduces metastasis (143). Cancer cells have the ability to uptake and metabolize lactate, allowing for proliferative capacity in lactate-rich environments (144, 145). Lactate accumulation has been shown to have an inhibitory effect on cytotoxic CD8⁺ T cells. However, the transcription factor FoxP3 induces oxidative phosphorylation, giving FoxP3⁺ Tregs an advantage over cytotoxic CD8⁺ T cells in high-lactate environments (146) (Figure 2). Therefore, the interaction between osteoblasts and metastatic seeds may not be limited to increased proliferation, but could also aid in the evasion of immune surveillance by providing an environment unsuitable for cytotoxic interactions while preserving immunosuppressive function. Osteoblasts express CXCL12, which allows for the homing of HSCs, Tregs, and metastatic seeds to the endosteum (Figure 2). Recruiting these cell types into an environment rich in lactate may further enhance metastatic targeting to the immune-privileged V. (15. **AUTHOR: Please clarify "immune-privileged V".**)

In summary, multiple cell types in the bone microenvironment may cooperate to establish an immunosuppressive environment. This environment is evolutionarily conserved due to the need for hematopoiesis, but may coincidentally facilitate the survival of DTCs and micrometastases. Future studies are needed to dissect the molecular mechanisms underlying DTC-immune system crosstalk and to identify actionable targets for therapeutic interventions.

Bone remodeling and repair impact metastatic colonization

In addition to their unique immune properties, bone and BM are also remarkable in their quick turnover. Bone is remodeled constantly, with old bones removed by osteoclasts and new bones deposited by osteoblasts, resulting in roughly 5%–25% of a total skeleton being replenished every year (147). Hematopoiesis is also a perpetual process generating new blood and immune cells, and 1% of hematopoietic cells are replaced every day. These activities accelerate during tissue repair and may also perturb the seeding and colonization of metastatic seeds. In this section, we summarize knowledge related to bone remodeling and repair and illustrate the potential link to metastasis.

Bone remodeling and repair. Adult bone is continually remodeling to respond to the changing mechanical needs of the body due to aging and environmental perturbations, and to address stress-induced microscopic damages (26, 148, 149). This complex process is regulated by local paracrine and systemic factors, such as calcium and the hormonal regulators PTH, calcitonin, and estrogen (148, 150). Local factors influence bone remodeling by acting on osteoblasts and osteoclasts, including BMPs, TGF- β , and WNTs (151, 152). When bones fracture, the first event that occurs is the formation of a hematoma by clotting blood (153, 154). This is followed by chondrocytes, the cartilage-producing cells of the

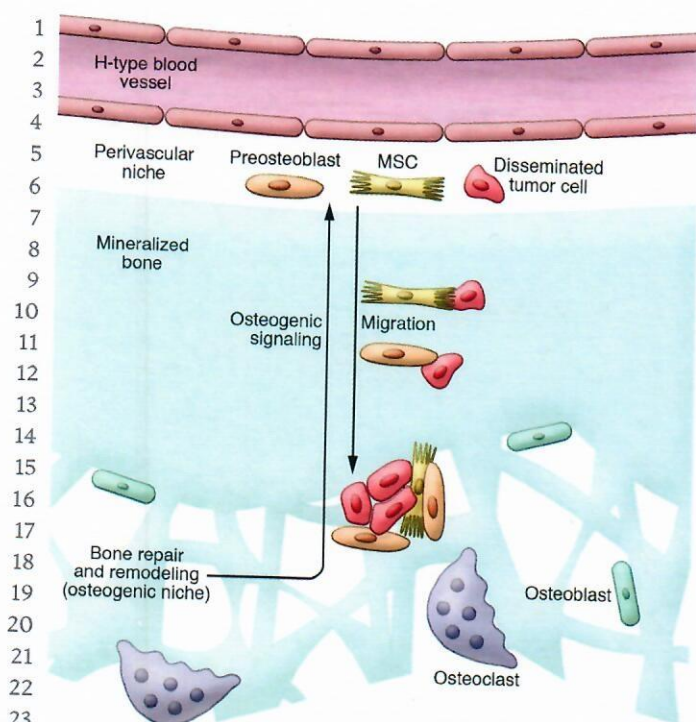


Figure 3. A potential connection between bone repair/remodeling and bone colonization of DTCs. One process that bridges different bone marrow niches is bone repair/remodeling. In this process, MSCs migrate from their perivascular origin to areas of bone repair or turnover. Recent evidence suggests that adhesion and collective migration with osteogenic cells enable DTCs to “ride” MSCs and traffic from the perivascular niche to the osteogenic niche in response to osteogenic signals. This may couple bone homeostasis and metastatic colonization.

bone, infiltrating the hematoma and laying down a callus between the broken bone surfaces (154, 155). Osteoblasts and chondrocytes lay down cartilage and hydroxyapatite, respectively, that slowly harden this callus (154, 156). Over the following weeks, both osteoclasts and osteoblasts remove dead bone and debris from the area, and lay down even more new mineral. These osteoblasts are derived from marrow MSCs and migrate into the callus in order to accomplish this repair (157). MSCs respond to chemotactic signals during the callus formation, causing them to migrate toward the sites of repair and undergo osteogenic differentiation (158). Once the callus is totally ossified, it is replaced with trabecular bone, and repair is complete, with new cortical bone forming on the exterior surface (152).

Exploitation of bone homeostasis by cancer cells. DTCs and fully pathogenic metastases may exploit bone homeostasis, remodeling, and repair in a multitude of ways. As noted earlier, DTCs residing in the perivascular niche after extravasation are nearby or in contact with perivascular MSCs and are held in quiescence by these pericytes/NG2⁺ MSCs as well as by endothelial cells (159–162). Therefore, relocation from the perivascular niche may be advantageous for the DTCs to progress to colonization. Fractures and subsequent remodeling stimulate the chemotaxis of MSCs toward the injured or remodeling site, where local factors subsequently promote MSC osteogenesis (158, 163). This process

may coincide with migration of DTCs from the perivascular niche, where they are kept dormant, to the osteogenic niche, which supports their proliferation (Figure 3). New evidence suggests that BCa cells can attach to and migrate with osteogenic cells via specialized protrusions, and this collective migration phenomenon may facilitate such a transition (164).

Thus, osteogenic activity in bone remodeling or in the soft callus, and later hard callus, stage of repairs is an opportunity for BCa DTCs to encounter and be supported by the osteogenic niche. The same process may also provide access to active osteoclasts and therefore (16. **AUTHOR: Is a word(s) missing here? Please rephrase to clarify this sentence.**) the transition toward late-stage osteolytic vicious cycle as osteoclasts are recruited to clear the soft callus and resorb the unneeded bone (17. **AUTHOR: Changed “bones” to “bone”. Is your meaning as intended?**) in normal remodeling.

Clinical interventions

A majority of BoMet treatments are palliative, with limited ability to eradicate the bone lesions entirely (165). However, much progress has been made in recent years. BoMet treatments, like many cancer treatments, can be chemical or radiological in nature. Below, we discuss some of the current BoMet treatments.

Antiresorption therapies. Bisphosphonates are a class of drugs originally developed for the treatment of osteoporosis (166). These drugs are chemically similar to inorganic phosphate and thus have high affinity for the calcium ions in hydroxyapatite (167). As calcium is released during bone resorption, bisphosphonates are especially targeted to areas of high osteoclast activity/bone resorption (168). These drugs act by disrupting the key osteoclast enzymes, farnesyl diphosphate synthases (169), thereby impairing their recruitment and differentiation and promoting osteoclast apoptosis (170). Zoledronic acid is the most potent bisphosphonate and was more recently developed to be more potent than earlier-generation bisphosphonates by modifying side chain functional groups (171, 172). Zoledronic acid improves outcomes compared with palliative maintenance of BoMet, with studies showing significant decreases in skeletal-related events and increased overall survival in BCa patients (173). Interestingly, in postmenopausal women, bisphosphonates exhibit significant efficacy in the adjuvant setting in terms of bone recurrence as well as overall survival (174). This observation cannot be explained by the inhibition of osteoclasts, as the vicious cycle has not started during adjuvant therapies. However, it is consistent with the fact that bisphosphonates reduce bone turnover (175), a potential driving force of DTCs to relocate between BM niches as previously discussed.

More recently, denosumab, a RANKL inhibitor/neutralizing antibody, has also been demonstrated to prevent osteoclast differentiation (176). This activity decreases osteolytic metastases in BCa patients (177). A reduction in skeletal-related events and clinical fracture risk was also seen in prostate cancer patients (178) as well as some other solid cancer types (179).

Radiation. External beam radiation treatments can be problematic for treatment of bone metastases because it is difficult to penetrate the mineralized bone tissue. However, radiation treatment is used in palliative care/bone-pain relief and the prevention of skeletal complications (180, 181). Ionizing beam radiation is able

to both kill tumor cells and decrease osteoclast activation, both of which can alleviate metastasis-associated pain (182, 183). Other options include radiopharmaceuticals that produce β or α particles like samarium and radium-223 (Rad-223) (18. **AUTHOR: Changed “Rad-233” to “Rad-223”. OK?**), respectively (184–187). Samarium and strontium have been used for prostate cancer lesions, again for pain management and palliative care, but survival benefits are minimal (188, 189). However, Rad-223 therapy has been shown to prolong survival and increase quality of life in patients with advanced cancer (184, 190). As radium localizes to the mineralized bone tissue, recent work shows it to be much more effective when the initial cell load is smaller, and a higher proportion of tumor cells are physically closer to the bone surface. These data suggest that Rad-223 treatment may be more effective when given to patients at an earlier stage (191, 192).

Immunotherapy. The use of immunotherapies aims to provide patients with more targeted approaches to treat cancer. Cancer cells or immune cells can express checkpoint inhibitor molecules that inhibit CD8⁺ cytotoxic T cells. A study looking at the effects of immune checkpoint blockade in combination with chemotherapy in triple-negative BCa demonstrated the ability of immune checkpoint blockade to prolong progression-free survival (193). However, in patients with BoMet, this prolonged survival was abrogated. Although this may be due to these patients suffering from additional metastatic burden in other organs, it may also indicate that the BM microenvironment influences patient responses to immunotherapy. TGF- β has been implicated in the effectiveness of immune checkpoint blockade therapy in metastatic urothelial cancer, where a high TGF- β signature resulted in a lower response to immune checkpoint blockade (194). This may have implications in the BM, where TGF- β is produced at various stages of metastasis.

Conclusions

When DTCs arrive in the bone and BM, they encounter a new environment composed of residential cells that are actively maintaining homeostasis of the skeletal and hematopoietic systems.

These cells are highly organized and constitute distinctive niches that together fulfill diverse functions. Interactions with different niches may dictate the cellular fates and therapeutic responses of DTCs and microscopic metastases. Cancer cells may exploit the niches' normal roles, including protection from aberrant immune activation and remodeling/repair of bones, to facilitate metastatic progression. Therefore, identification of the cancer-niche cross-talk pathways, especially those involved in immunosurveillance and tissue repair, may lead to novel mechanistic insights and therapeutic targets.

There are challenges and opportunities in our further investigations of BM niches in metastatic colonization. Bone-resident cells apparently of the same type may actually be heterogeneous with regard to the location, lineage, and functions. This is exemplified by pericytes/MSCs (195, 196) and endothelial cells (197), which may reconcile the seemingly contradictory findings (e.g., refs. 81, 87). Future studies are needed for more precise definition of various niches. To this end, single-cell transcriptomic or proteomic analyses that preserve the spatial information may be highly valuable. It should be noted that fast progress has been made in dissecting and targeting BM niches in blood cancers (198). Systematic comparisons of BM niches between metastatic solid cancers and hematological malignancies will likely provide interesting mechanistic insights. In particular, therapies targeting the niches of the latter may inform future clinical studies of the former.

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