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PRINCIPAL INVESTIGATOR: Xiang Zhang

CONTRACTING ORGANIZATION: Baylor College of Medicine, Houston, TX

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14. ABSTRACT Bone metastases more frequently occur to estrogen receptor positive (ER+) breast cancer. Thus, it is imperative to understand how disseminated ER+ cancer cells escape therapies in distant organs and to identify therapies that can eliminate these cells. Supported by the DoD Era of Hope Award, my laboratory developed a series of models and techniques to investigate cancer-bone interaction at a single cell resolution. We have also elucidated an epigenomic reprogramming effect elicited by the bone microenvironment that leads to endocrine resistance. Importantly, this represents a mechanism fundamentally distinct from classic clonal evolution process. In this application, we aim to expand our research to understand the epigenetic mechanisms underlying the abovementioned effects. More importantly, we wish to identify therapeutic strategies that can reverse the endocrine resistance and prevent recurrences of ER+ breast cancer. It is important to note that multiple lines of evidence have emerged and suggest that many metastases are actually seeded by other metastases, rather than primary tumors. Two thirds of bone-only metastases are followed by appearance of metastases in multiple other organs. Our unpublished studies also demonstrated that bone microenvironment may invigorate disseminated breast cancer cells for further metastasis. Thus, our research focusing on bone metastasis may indeed generate broader impact and lead to reduction of metastases in other organs as well. Taken together, the proposed research represents a move beyond incremental advancement, and will likely lead to new metastatic and adjuvant therapies. Our study will demonstrate how epigenomic adaptation drives cancer evolution and alter therapeutic responses together with clonal selection, provides insights into the clinical enigma of ER+ metastatic recurrences despite endocrine therapies, and leads to reduction of metastasis-related breast cancer mortality.					
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1. Introduction

Metastasis to distant organs is the major cause of breast cancer-related death. Bone is the most frequent destination of metastasis. In its advanced stage, bone metastasis is driven by the paracrine crosstalk among cancer cells, osteoblasts, and osteoclasts, which together constitute an osteolytic vicious cycle. Specifically, cancer cells secrete molecules such as PTHrP, which acts on osteoblasts to modulate the expression of genes including RANKL and OPG. The alterations of these factors in turn boost osteoclast maturation and accelerate bone resorption. Many growth factors (e.g., IGF1) deposited in the bone matrix are then released, and reciprocally stimulate tumor growth. This knowledge has profoundly deepened our understanding of bone metastasis. However, several aspects of bone metastases remain poorly understood.

Bone is the most frequently affected organ by ER+ breast cancer, which is usually luminal-like. Compared to the basal-like subtype, luminal A/B breast cancer exhibits a 2.5-fold increased frequency of bone metastasis, but a 2.5-fold decreased frequency of lung metastasis. Bone metastases of luminal-like breast cancer are often late-onset, occurring beyond 5 years after surgery. Current tumor-intrinsic biomarkers based on primary tumors can predict recurrences within 5 years, but cannot predict late-onset recurrences after 5 years, suggesting that the capacity of developing late-onset metastasis is not encoded in cancer cells. Thus, there appear to be unique interactions between the bone microenvironment and ER+ disseminated tumor cells that allow them to survive adjuvant endocrine therapies and persist for a prolonged time.

Our knowledge also remains scarce about how the bone microenvironment affects ER+ breast cancer cells in terms of key signaling pathways (e.g., ER signaling itself), therapeutic responses, and the evolution process. The major obstacle is the lack of ER+ bone metastasis models. Supported by the DoD Era of Hope Award, my laboratory developed a series of models and techniques to investigate cancer-bone interaction at a single cell resolution. We have also elucidated an epigenomic reprogramming effect elicited by the bone microenvironment that leads to endocrine resistance and other unexpected therapeutic effects. In this application, we aim to expand our research to understand the epigenetic mechanisms underlying the abovementioned effects. Our goal is to identify therapeutic strategies that can reverse the endocrine resistance and prevent recurrences of ER+ breast cancer. We hypothesize that the direct and paracrine interactions between ER+ breast cancer cells and the osteogenic bone microenvironment elicit epigenetic adaptation, a process independent of clonal selection and leading to persistent endocrine resistance. Therapeutic blockade of the microenvironment-induced epigenomic reprogramming can reverse the resistance and reduce subsequent metastatic recurrences. To test the hypothesis, we will pursue two specific aims: 1) further dissect molecular mechanisms underlying the epigenomic reprogramming process induced by the bone microenvironment, and 2) to identify low-toxicity and efficient therapeutic strategies that can be added to standard-of-care treatments to eliminate bone micrometastases and prevent overt metastatic recurrences.

Our study will demonstrate how epigenomic adaptation drives cancer evolution and alter therapeutic responses together with clonal selection, provides insights into the clinical enigma of ER+ metastatic recurrences despite endocrine therapies, and leads to reduction of metastasis-related breast cancer mortality.

2. Keywords:

Metastasis, microenvironment, drug discovery, therapeutic resistance, micrometastases, endocrine resistance, the osteogenic niche, EZH2

3. Accomplishment

Because of the lab access restrictions during pandemic, we have reprioritized all experiments. A couple of sub-tasks were not carried out as planned (see details below). We will try our best to catch up in the coming grant years.

Task 1.1: To identify additional pathways that may contribute to the endocrine resistance in the bone microenvironment in parallel with EZH2 (All work will be in vitro)

During the pandemic and the lab shutdown, we performed a series of computational analyses to identify pathways that may be parallel to EZH2 in mediating endocrine resistance of bone metastasis. The FGFR pathway is upstream of EZH2 as we showed in our initial application as well as recent publications (see Products). We asked whether there are other downstream pathways that may also mediate the phenotypic plasticity and endocrine resistance observed in the bone microenvironment. Toward this end, we identified genes that are significantly correlated expression of FGFR1, FGFR2, FGFR3, and FGFR4 in TCGA breast cancer datasets. The top genes were then used for gene ontology enrichment analysis. Interestingly, the four FGFRs are associated with largely different pathways (Figure 1). The association between FGFR1 and cell adhesion pathways may indicate its important roles in bone metastasis, especially the direct cell-cell interactions between cancer cells and osteogenic cells, as we previously showed (1-3). Future studies will further dissect the regulation of cell adhesion by FGFR1.

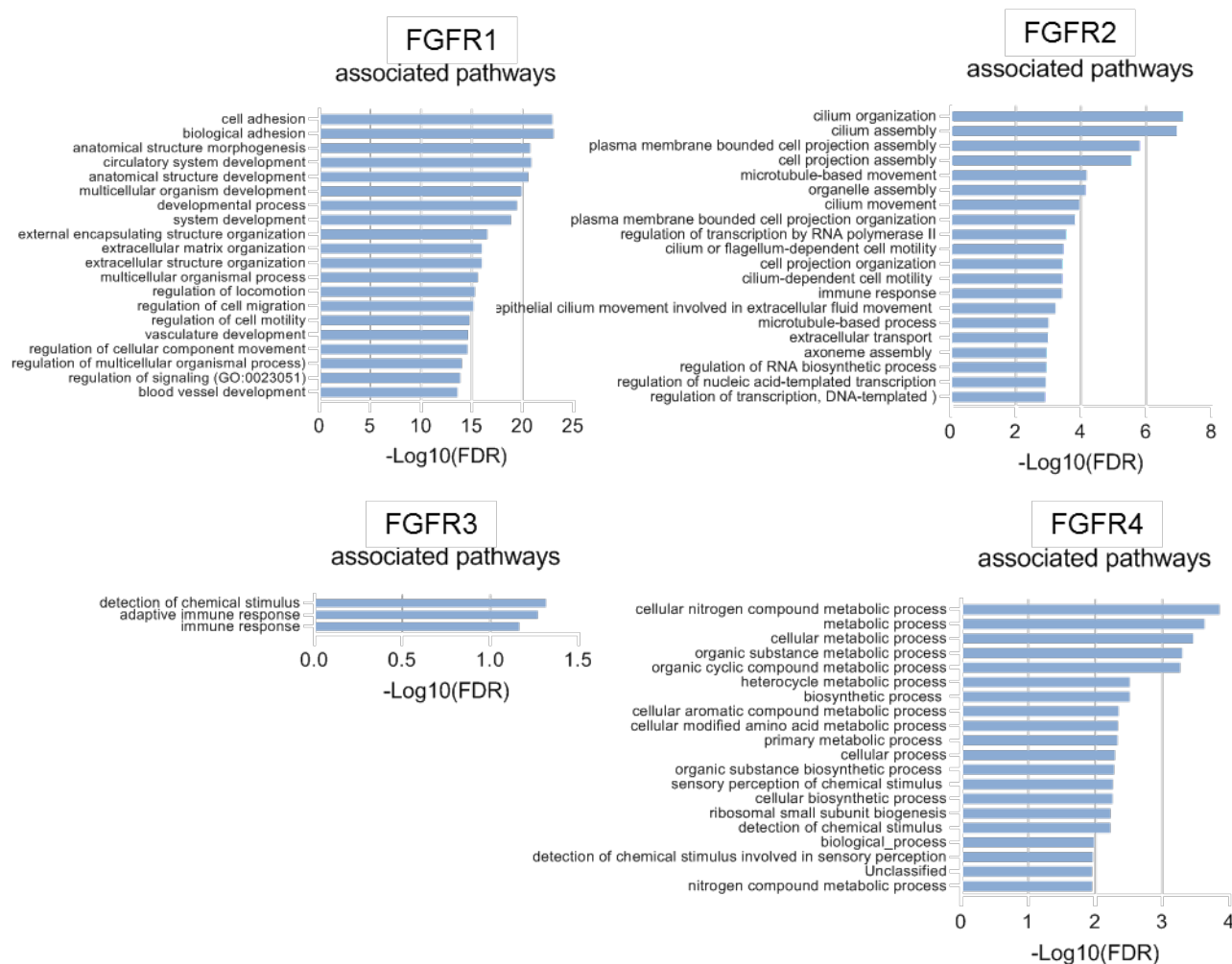


Figure 1: Identification of additional pathways that may contribute to the endocrine resistance in the bone microenvironment in parallel with EZH2. Genes that correlate with each of the four FGFRs were identified in TCGA, and were subjected to the Gene Ontology Enrichment Analysis. The top-scored pathways were presented with $-\log_{10}(\text{FDR})$ indicated.

Sub task 1.1.1: Using 3D in vitro co-cultures, we will test if BGJ398 and sitravatinib can abolish osteogenic cell-conferred endocrine resistance. (Month 1-3)

We have completed testing the combined effects of BGJ398 and fulvestrant on three ER+ cancer cell lines in the co-culture with human fetal osteoblasts (hFOB). As expected, BGJ398 treatment abolished the resistance to fulvestrant both in simple co-cultures and in bone-in-culture array (BICA) (Figure 2). The latter is an ex vivo

system that recapitulate many aspects of the bone microenvironment. The same experiments using sitravatinib will be performed in the coming grant year.

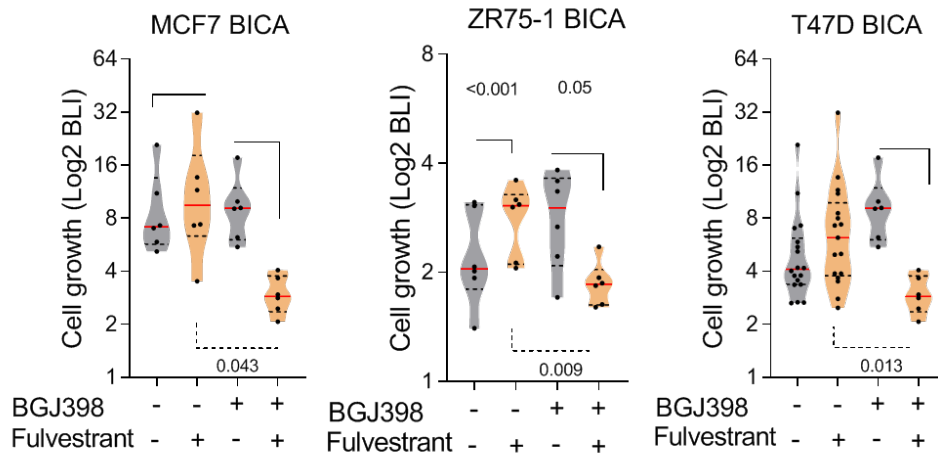


Figure 2. BGJ398 treatment synergizes with fulvestrant in bone-in-culture array (BICA). Three ER+ cancer cell lines were subjected to intra-iliac artery injection to introduce experimental bone metastases. The metastasis-carrying bones were then extracted and fragmented to establish arrays of bone fragments containing cancer cells. Our previous studies demonstrated that cancer-bone interactions are well preserved in this platform for up to six weeks (1). Herein, the combined treatment exhibited superior efficacies, supporting that inhibition of FGFR signaling abolished endocrine resistance in bone-like microenvironment.

Sub task 1.1.2: We will combine BGJ398 or sitravatinib with tezametostat, either concurrently or sequentially, and determine if the two types of treatments may together achieve a better synergy with endocrine therapies. (Month 3-6)

This sub-task has not been started as planned. We will pursue it in the coming grant period.

Sub task 1.1.3: To assess the potential toxicity of combining multiple drugs, we will use non-transformed human mammary epithelial cells (HMECs) as a control, and prioritize drug combinations that selectively eliminate cancer cells but not non-transformed cells in co-cultures. (Month 3-6)

This sub-task has not been started as planned. We will pursue it in the coming grant period.

Sub task 1.1.4: Complementary genetic experiments will be conducted using inducible shRNAs against FGFR1, PDGFR β , and EZH2 to ensure that drug effects are achieved through the expected target proteins. (Month 6-18) Instead of shRNAs, we decided to use recombinant proteins or neutralizing antibodies to manipulate FGF2 due to the shorter experiment turnover time. Indeed, these treatments generate significant impact on cancer cells' responses to fulvestrant, further supporting the conclusion generated in Sub task 1.1.1 (Figure 3). In the coming grant period, we will continue to test PDGFR β and EZH2 using genetic or antibody approaches to validate them as drug targets mediating observed endocrine resistance.

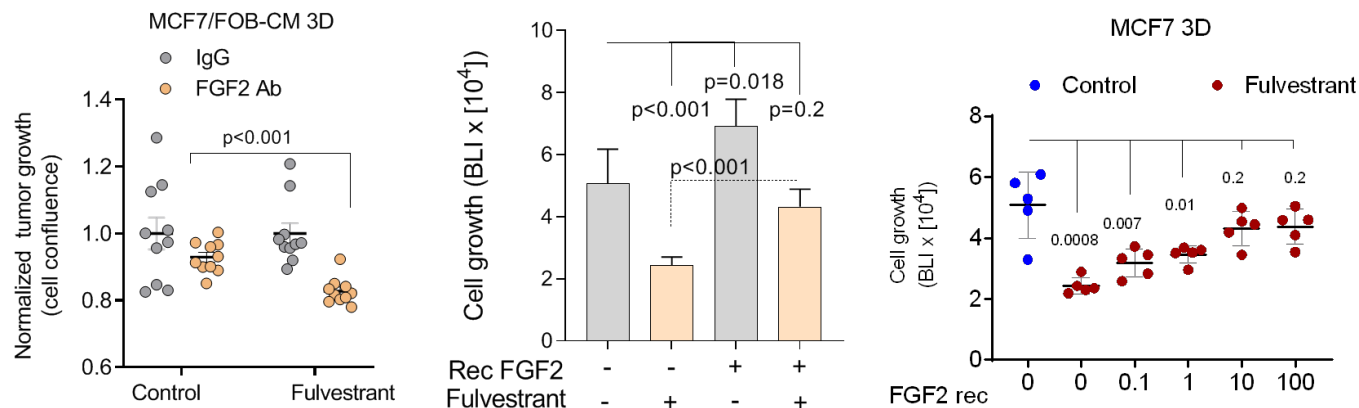


Figure 3. Treatment of FGF2 neutralizing antibody (FGF2 Ab) or recombinant (rec) FGF2 protein abolishes or increases resistance to fulvestrant in 3D cell cultures, respectively.

Sub task 1.1.5: We will ask if overexpression of exogenous EZH2 may overcome BGJ398 and sitravatinib and restore endocrine resistance, and if knockdown of EZH2 may reverse the effects of FGF2 and PDGF-DD on cancer cell growth and endocrine resistance. (Month 12-18)

This sub-task has not been started as planned. We will pursue it in the coming grant period.

Task 1.2: To delineate the temporal course of epigenomic programming, and determine therapeutic window to reverse the endocrine resistance. (Month 6-18)

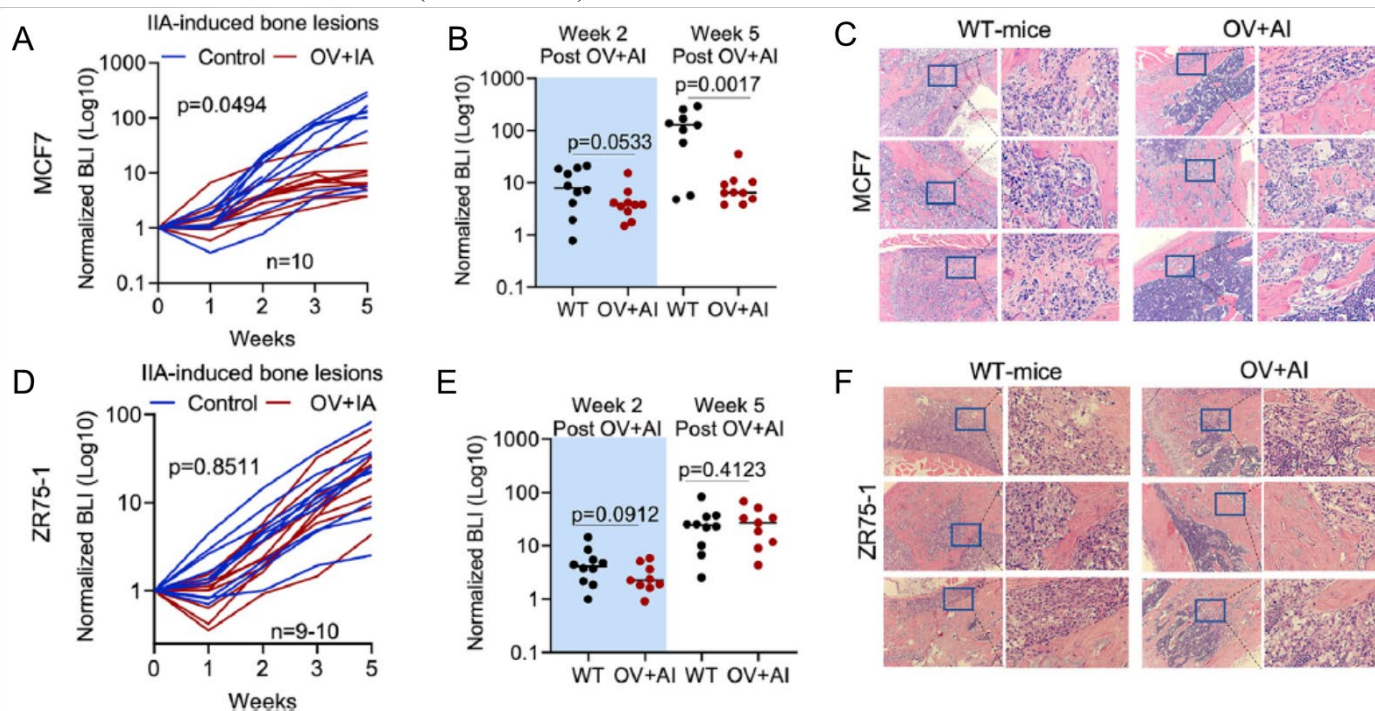


Figure 4. Time windows of bone microenvironment-induced endocrine resistance. **A.** Growth curve showing response of IIA-induced MCF7 BoMs to estrogen depletion. Ovariectomized (OV) mice were additionally treated with Letrozole (OV+AI), daily. Results are based on bioluminescence. (n=10 mice); P value: Two-Way ANOVA. **B.** Dot plot showing BoM growth in wild-type (WT) and OV+AI mice at week 2 and week 5 post tumor transplantation; n=10 mice. P value: two-tailed unpaired Student's t-test. **C.** H&E staining showing MCF7 metastatic lesions in wild-type (WT) and "OV+AI" groups. **D.** Growth curve depicting the response of ZR75-1-derived BoM as in A. (n=9-10 mice); P value: Two-Way ANOVA. **E.** Dot plot showing statistical growth differences in ZR75-1 as in I. **F.** H&E staining of ZR75-1 metastatic lesions as in D.

As planned, we examined responses of ER⁺ cancer cells to estrogen deprivation in bone microenvironment. Combined ovariectomy and letrozole treatment could significantly impede orthotopic tumor growth in both MCF-7 and ZR75-1 models, but failed to reduce bone metastatic colonization at early time points (Figure 4A-4F). The response to estrogen deprivation was partially recovered in MCF-7 cells after Week 3 (Figure 4A-4C),

further supporting that the resistance may be reversible as metastases further progress. However, ZR75-1 bone lesions remained resistant at later time points (Figure 4D-4F). Hence, not all bone metastases restore endocrine sensitivity. In ZR75-1, despite the partial reversion of ER expression in macrometastases, PR expression remained repressed. Thus, downstream ER signaling may not recover together with ER expression during BoM progression.

Task 2.1: To characterize the pharmacokinetics and toxicity of tezametostat, BGJ398, and/or sitravatinib in the context of bone metastasis. This will be performed in collaboration with the DMPK core of BCM. (Month 1-12)

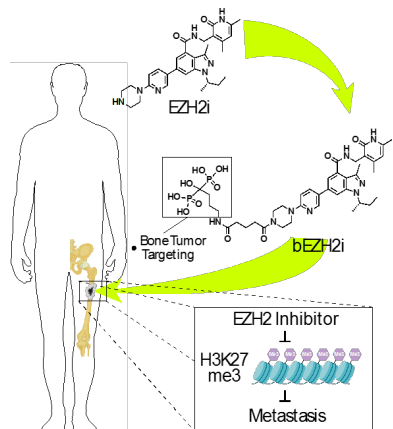


Figure 5. Strategy to overcome poor bioavailability of EZH2 inhibitors in bone microenvironment.

Due to the pandemic, BCM Core facilities were subjected to restrictions. In the meantime, we learned from the literature that the bioavailability of many chemical inhibitors in bone, including those targeting EZH2, are poor. Therefore, instead of merely measuring the pharmacokinetics of tezametostat, we proactively sought to improve the pharmacokinetics. In collaboration with Dr. Han Xiao at Rice University, we have developed an alternative plan to increase the bioavailability of EZH2 inhibitors in bone. Specifically, we conjugated an EZH2 inhibitor with bisphosphonates. The latter, as widely known, are bone-seeking chemicals that are not only enriched in the bone microenvironment but also targeting osteoclasts for apoptosis. In the coming grant period, we will characterize this new compound drug and test its efficacy on bone metastasis. Although this new plan deviates from the original proposal, it focuses on the same hypothesis and will more likely lead to novel findings and development of new drugs.

Task 2.2: To identify adjuvant therapeutic strategies against the bone microenvironment-induced endocrine resistance and prevent overt bone metastases.

Subtask 2.2.1: Experimental bone colonization assays. (Month 12-24)

Not started yet.

Subtask 2.2.2: Spontaneous bone metastasis assays. (Month 18-36)

Not started yet.

Task 2.3. To determine if the same mechanism of endocrine resistance persists in overt bone metastases and anti-EZH2 treatment can be used at the metastatic setting. (Month 24-36)

Not started yet.

4. Impact

Our study will demonstrate how epigenomic adaptation drives cancer evolution and alter therapeutic responses together with clonal selection, provides insights into the clinical enigma of ER+ metastatic recurrences despite endocrine therapies, and leads to reduction of metastasis-related breast cancer mortality.

5. Changes/Problems

The Covid 19 pandemic has significantly impacted our ability to access lab and animal facilities and core services of college. However, we have still made significant progress, and will catch up with the proposed progress in the coming year.

Instead of simply characterizing the pharmacological properties of EZH2 inhibitors, we have established collaborations to actually improve bioavailability of EZH2 inhibitors in the bone (Figure 5). This is a significant step more advanced compared to what was originally proposed. We are confident that this change will lead to stronger and faster translation of our research product.

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, 56(8):1100-1117. PMCID: PMC8062036.
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5. Tian Z, Wu L, Yu C, Chen Y, Xu Z, Bado I, Loreda 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 (Of note, this grant will focus on conjugate bisphosphonate to anti-Her2 antibodies for elimination of bone micrometastases, and therefore, will not overlap with the current grant).

7. Participants & Other Collaborating Organizations

Name:	Ling Wu
Project Role:	Postdoctoral Fellow
Researcher Identifier:	N/A
Nearest person month worked	12
Contribution	Dr. Wu specialize in bone metastasis research techniques and is leading the proposed experiments.

Name:	Xiang Zhang
Project Role:	PI/PD
Researcher Identifier:	N/A
Nearest person month worked	0.912

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.

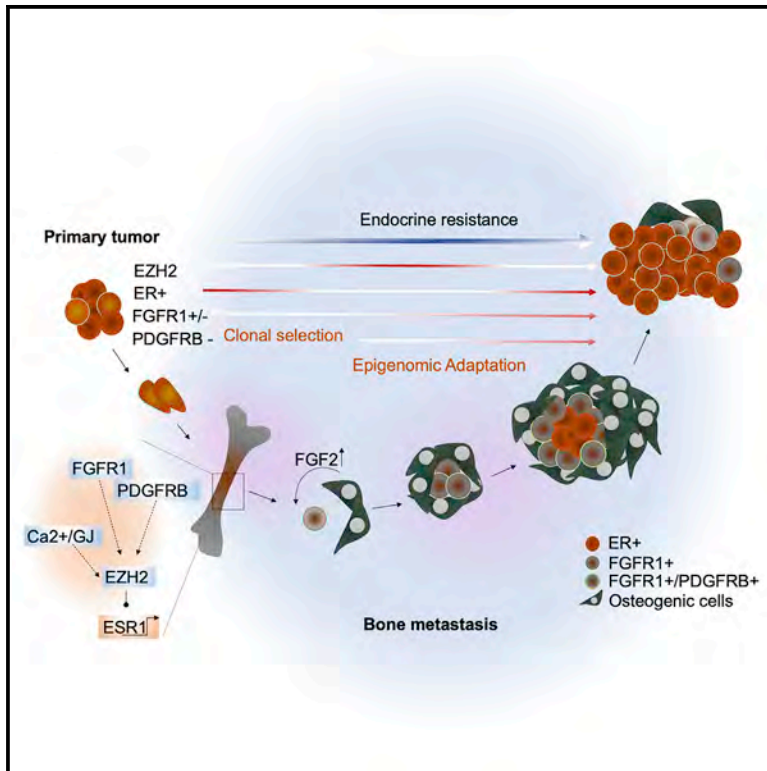
Reference:

1. Wang H, Tian L, Goldstein A, Liu J, Lo HC, Sheng K, Welte T, Wong STC, Gugala Z, Stossi F, Zong C, Li Z, Mancini MA, Zhang XH. Bone-in-culture array as a platform to model early-stage bone metastases and discover anti-metastasis therapies. *Nat Commun.* 2017;8:15045. Epub 2017/04/22. doi: 10.1038/ncomms15045. PubMed PMID: 28429794; PMCID: PMC5413944.
2. Wang H, Tian L, Liu J, Goldstein A, Bado I, Zhang W, Arenkiel BR, Li Z, Yang M, Du S, Zhao H, Rowley DR, Wong STC, Gugala Z, Zhang XH. The Osteogenic Niche Is a Calcium Reservoir of Bone Micrometastases and Confers Unexpected Therapeutic Vulnerability. *Cancer Cell.* 2018;34(5):823-39 e7. Epub 2018/11/14. doi: 10.1016/j.ccell.2018.10.002. PubMed PMID: 30423299; PMCID: PMC6239211.
3. Wang H, Yu C, Gao X, Welte T, Muscarella AM, Tian L, Zhao H, Zhao Z, Du S, Tao J, Lee B, Westbrook TF, Wong ST, Jin X, Rosen JM, Osborne CK, Zhang XH. The osteogenic niche promotes early-stage bone colonization of disseminated breast cancer cells. *Cancer Cell.* 2015;27(2):193-210. Epub 2015/01/21. doi: 10.1016/j.ccell.2014.11.017. PubMed PMID: 25600338; PMCID: PMC4326554.

Developmental Cell

The bone microenvironment increases phenotypic plasticity of ER⁺ breast cancer cells

Graphical abstract



Authors

Igor L. Bado, Weijie Zhang, Jingyuan Hu, ..., Zbigniew Gugala, Zhandong Liu, Xiang H.-F. Zhang

Correspondence

xiangz@bcm.edu

In brief

Bado et al. demonstrate that the osteogenic niche enhances the phenotypic plasticity of metastatic ER⁺ breast cancer cells through an EZH2-mediated epigenomic reprogramming. The resulting spatiotemporal heterogeneity of ER expression leads to increased stemness and endocrine resistance. Targeting EZH2 restores endocrine sensitivity and synergizes with standard anti-ER therapies.

Highlights

- ER⁺ breast cancer cells transiently reduce ER expression in bone micrometastases
- Interaction with osteogenic cells causes reversible ER loss and endocrine resistance
- Epigenomic mechanisms lead to ER reduction, cancer stemness, and cellular plasticity
- EZH2 mediates the impact of bone and is a target to alleviate endocrine resistance



Article

The bone microenvironment increases phenotypic plasticity of ER⁺ breast cancer cells

Igor L. Bado,^{1,2,3,11} Weijie Zhang,^{1,2,3,11} Jingyuan Hu,^{4,5,6} Zhan Xu,^{1,2,3} Hai Wang,^{1,2,3} Poonam Sarkar,⁵ Lucian Li,^{5,6} Ying-Wooi Wan,^{5,6,7} Jun Liu,^{1,2,3} William Wu,⁷ Hin Ching Lo,^{1,2,3} Ik Sun Kim,^{1,2,3} Swarnima Singh,^{2,3} Mahnaz Janghorban,^{2,3} Aaron M. Muscarella,^{1,2,3} Amit Goldstein,^{1,2,3} Purba Singh,^{1,2,3} Hyun-Hwan Jeong,⁸ Chaozhong Liu,^{5,6} Rachel Schiff,^{1,2} Shixia Huang,³ Matthew J. Ellis,^{1,2,3} M. Waleed Gaber,⁵ Zbigniew Gugala,⁹ Zhandong Liu,^{6,7} and Xiang H.-F. Zhang^{1,2,3,10,12,*}

¹Lester and Sue Smith Breast Center, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA

²Dan L. Duncan Cancer Center, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA

³Department of Molecular and Cellular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA

⁴Program in Quantitative and Computational Biosciences, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA

⁵Department of Pediatrics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA

⁶Jan and Dan Duncan Neurological Research Institute, Texas Children's Hospital, Houston, TX 77030, USA

⁷Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA

⁸Center for Precision Health, School of Biomedical Informatics, the University of Texas Health Science Center at Houston, Houston, TX 77030, USA

⁹Department of Orthopedic Surgery and Rehabilitation, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555, USA

¹⁰McNair Medical Institute, Baylor College of Medicine, BCM600, One Baylor Plaza, Houston, TX 77030, USA

¹¹These authors contributed equally

¹²Lead contact

*Correspondence: xiangz@bcm.edu

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SUMMARY

Estrogen receptor-positive (ER⁺) breast cancer exhibits a strong bone tropism in metastasis. How the bone microenvironment (BME) impacts ER signaling and endocrine therapy remains poorly understood. Here, we discover that the osteogenic niche transiently and reversibly reduces ER expression and activities specifically in bone micrometastases (BMMs), leading to endocrine resistance. As BMMs progress, the ER reduction and endocrine resistance may partially recover in cancer cells away from the osteogenic niche, creating phenotypic heterogeneity in macrometastases. Using multiple approaches, including an evolving barcoding strategy, we demonstrated that this process is independent of clonal selection, and represents an EZH2-mediated epigenomic reprogramming. EZH2 drives ER⁺ BMMs toward a basal and stem-like state. EZH2 inhibition reverses endocrine resistance. These data exemplify how epigenomic adaptation to BME promotes phenotypic plasticity of metastatic seeds, fosters intra-metastatic heterogeneity, and alters therapeutic responses. Our study provides insights into the clinical enigma of ER⁺ metastatic recurrences despite endocrine therapies.

INTRODUCTION

Estrogen receptor-positive (ER⁺) breast cancer (BC) accounts for over 70% of all BCs, and after recurring, causes over 24,000 deaths per year in the US. Adjuvant endocrine therapies target ER and significantly reduce recurrences. However, 20%–40% of patients still develop metastases, often after a prolonged latency (Lim et al., 2012; Zhang et al., 2013). Thus, it is imperative to understand how ER⁺ cancer cells escape endocrine therapies in distant organs and to identify therapies to eliminate these cells.

Bone is the most frequently affected organ by ER⁺ BC, which is usually luminal-like subtype. Compared with the basal-like subtype, luminal BC exhibits a 2.5-fold increased frequency of

bone metastasis (BoM), but a 2.5-fold decreased frequency of lung metastasis (Kennecke et al., 2010; Smid et al., 2008). BoMs of luminal-like BC are usually late onset, occurring beyond 5 years after surgery. Current tumor-intrinsic biomarkers in primary tumors can predict recurrences within 5 years, but not beyond (Sgroi et al., 2013), suggesting that the capacity of developing late-onset metastasis may not be encoded in cancer cells. We hypothesize that unique interactions between the bone microenvironment (BME) and ER⁺ disseminated tumor cells (DTCs) may facilitate survival and therapeutic resistance.

Little is known about how the BME affects ER⁺ BC cells. There is a paradoxically high discordance rate of ER status between primary tumors and DTCs, suggesting loss of ER in DTCs

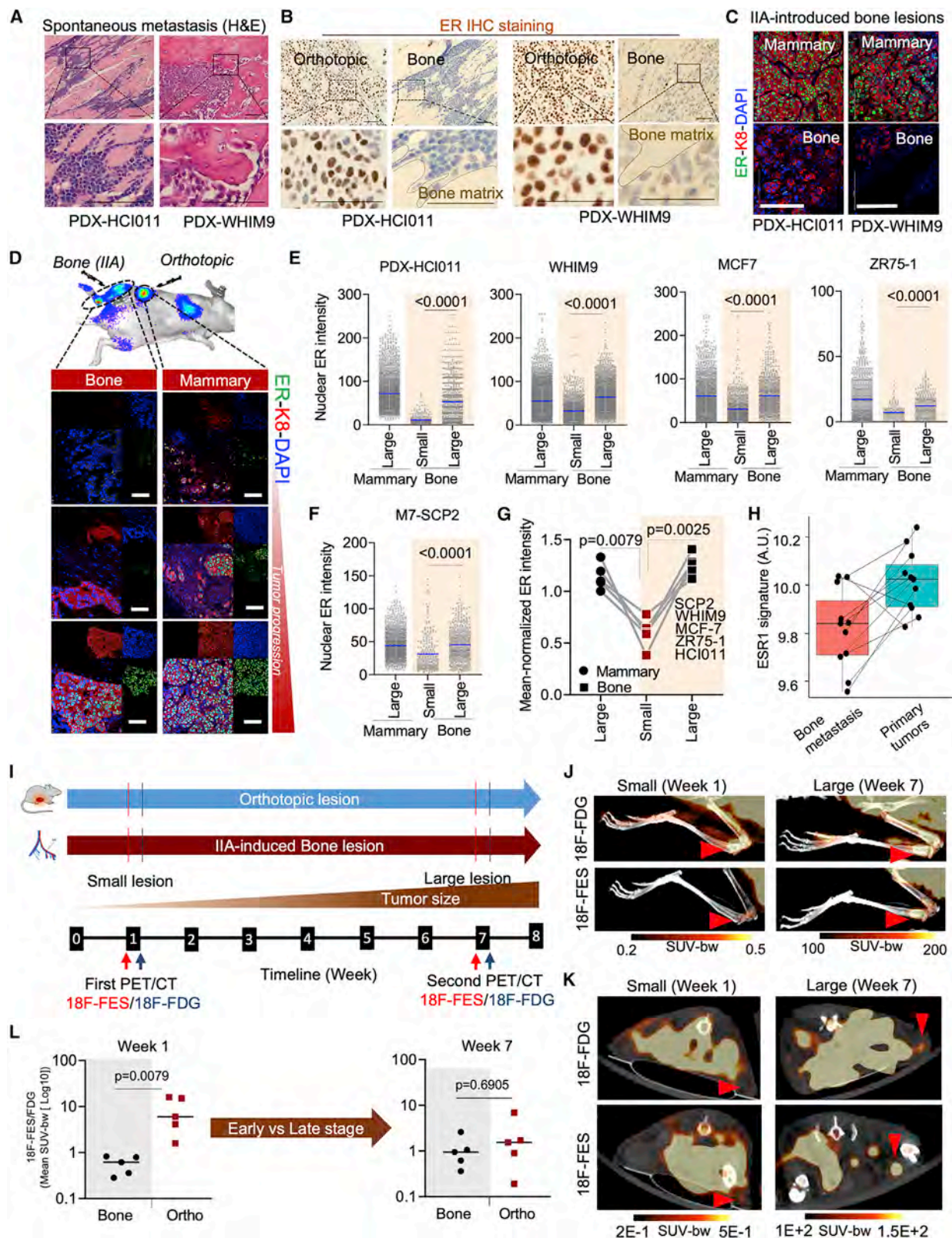


Figure 1. BME induces transient loss of ER expression in ER⁺ breast cancer cells

(A) H&E staining of spontaneous metastases of HCI011 and WHIM9 tumors to spine and hind limb, respectively. Scale bar: 100 μ m.

(B) Human-specific ER IHC staining are shown for spontaneous metastasis of HCI011 and WHIM9, respectively. Scale bar: 50 μ m.

(legend continued on next page)

(Fehm et al., 2008; Jäger et al., 2015). However, most overt BoMs (>85%) remain positive for ER (Hoefnagel et al., 2013), seemingly contradicting the DTC findings. ER⁺ BoMs can still respond to endocrine therapies, although resistance almost invariably develops.

We have previously developed a series of models and techniques to investigate cancer-bone interaction at a single-cell resolution. Herein, we aim to investigate the impact of BME on the ER signaling in ER⁺ BC cells.

RESULTS

BMMs transiently lose ER expression

To study how ER⁺ BC cells interact with BME, we identified two patient-derived xenograft models (PDXs), HCI011 and WHIM9, from patients with BoMs (Derose et al., 2011; Li et al., 2013). In immunodeficient mice, orthotopic HCI011 and WHIM9 tumors spontaneously metastasized to bones 5–6 months after surgeries (Figure 1A). Spontaneous BoMs exhibited reduced ER expression in both PDX models (Figure 1B). The BoM size positively correlated with an average intensity of nuclear ER by IHC staining (Figures S1A and S1B).

Next, we performed intra-iliac artery (IIA) injection of dissociated PDXs or established ER⁺ cell lines (MCF-7 and ZR75-1) to introduce experimental BoMs. This approach synchronizes the onset of colonization and enriches BMMs, thereby allowing quantitative examination of bone colonization of relatively indolent cancer cells at different stages (Figures 1C and 1D) (Yu et al., 2016). As in spontaneous BoMs, a strong correlation was found between ER expression and the size of IIA-induced BoMs (Figure S1C) but not orthotopic tumors (Figure S1D). When we classified BoMs by size, the expression of ER diminished in small lesions compared with mammary tumors but was restored in large lesions (Figure 1E). Thus, in both spontaneous and experimental BoM models, lesion size seems to be associated with ER expression (Figures 1E–1G).

Two possible mechanisms might explain the above association. First, genetically distinct ER^{low} and ER^{high} cancer cells may pre-exist, and the former progresses at a slower rate and form small lesions. Second, there may be a transient loss and a subsequent recovery of ER in ER⁺ cancer cells in BME. To

distinguish these, we collected four single-cell-derived populations from MCF-7 cells (M7-SCPs). Exome sequencing validated their genetic purity (Figure S1E). The expression of ER in M7-SCPs is still variable from cell to cell, although to a lesser degree compared with parental cells (Figure S1F), suggesting a baseline level of cellular plasticity among ER⁺ BC cells. Using an M7-SCP of the best clonality (M7-SCP2), we compared the ER expression between BMMs and macrometastases. Like parental MCF7, M7-SCP2 cells exhibited decreased ER expression in small BoMs (Figures 1F, S1G, and S1H). Taken together, multiple ER⁺ models supported that BME induces a loss of ER expression specifically in BMMs (Figure 1G).

The loss of ER was also observed in clinical specimens. In a study comparing patient-matched primary BCs and BoMs, ESR1 was found to be one of the top genes downregulated in BoMs (Priedigkeit et al., 2017). Gene set variation analysis (GSVA) further suggests that there is an even stronger downregulation of acute ER signaling (Figure 1H).

We next monitored the longitudinal alteration of ER signaling during BoM progression using positron emission tomography-computed tomography (PET-CT) imaging. A radiolabeled 18F-fluoroestradiol (18F-FES) PET/CT imaging strategy was adopted (Figures 1I and S1I–S1K) to measure the uptake of estrogen by tumors in parallel with glucose consumption (18F-FDG) (Kurland et al., 2017). We found a significant reduction in estrogen uptake in early lesions of BoM (Figure 1J) compared with mammary tumors (Figures 1K and S1I). The difference was reduced at a later time point (Figures 1J–1L), suggesting a reversible process.

An evolving barcode strategy to trace clonal evolution of ER⁺ BC cells

ER heterogeneity is often observed in the clinic. However, the phylogenetic relationship between ER⁺ and ER[−] cancer cells is not defined. We used an evolving barcode system (Kallhor et al., 2017, 2018) to trace the evolution of ER⁺ cancer cells in BME. This is a variant of the widely used CRISPR-Cas9 system. Here, the PAM site is mutated such that Cas9 can target the locus encoding guide RNAs (termed “home-guide RNAs” or hgRNAs). As a result, mutations are iteratively introduced to drive the evolution of hgRNAs. This provides an opportunity for multiple parallel lineage tracing in cancer.

(C) IF staining of ER, keratin 8 (K8), and DAPI in orthotopic (mammary) and IIA-induced BoM models of ER⁺ PDXs (HCI011 and WHIM9). Scale bars: 100 μ m.
(D) IF images of MCF7 cells following orthotopic and bone transplantation. Changes in ER expression are illustrated at different stages of tumor progression. Scale bars: 50 μ m.

(E) Dot plot depicting nuclear ER intensity of single cells (SCs) in orthotopic and BoM specimens from PDXs (HCI011, WHIM9) and cell lines (MCF7, ZR75-1), following IF staining as illustrated in (C and D) ($n = 3$ –6 mice per model). Error bars: mean $\pm$ SD.

(F) Dot plot depicting nuclear ER intensity of SCs in orthotopic and BoM specimens from M7-SCP2. Bone lesions were classified into “small” and “large” as defined in (E); ($n = 4$ mice). Error bars: mean $\pm$ SD.

(G) Connected scatterplots showing the mean-normalized ER intensity of all cancer models used from 1E to 1F. ($n = 5$ cell lines).

(H) Boxplot depicting changes in ESR1 early signature in matched BoM and primary specimens from BC patients (Github: <https://github.com/npriedig/>).

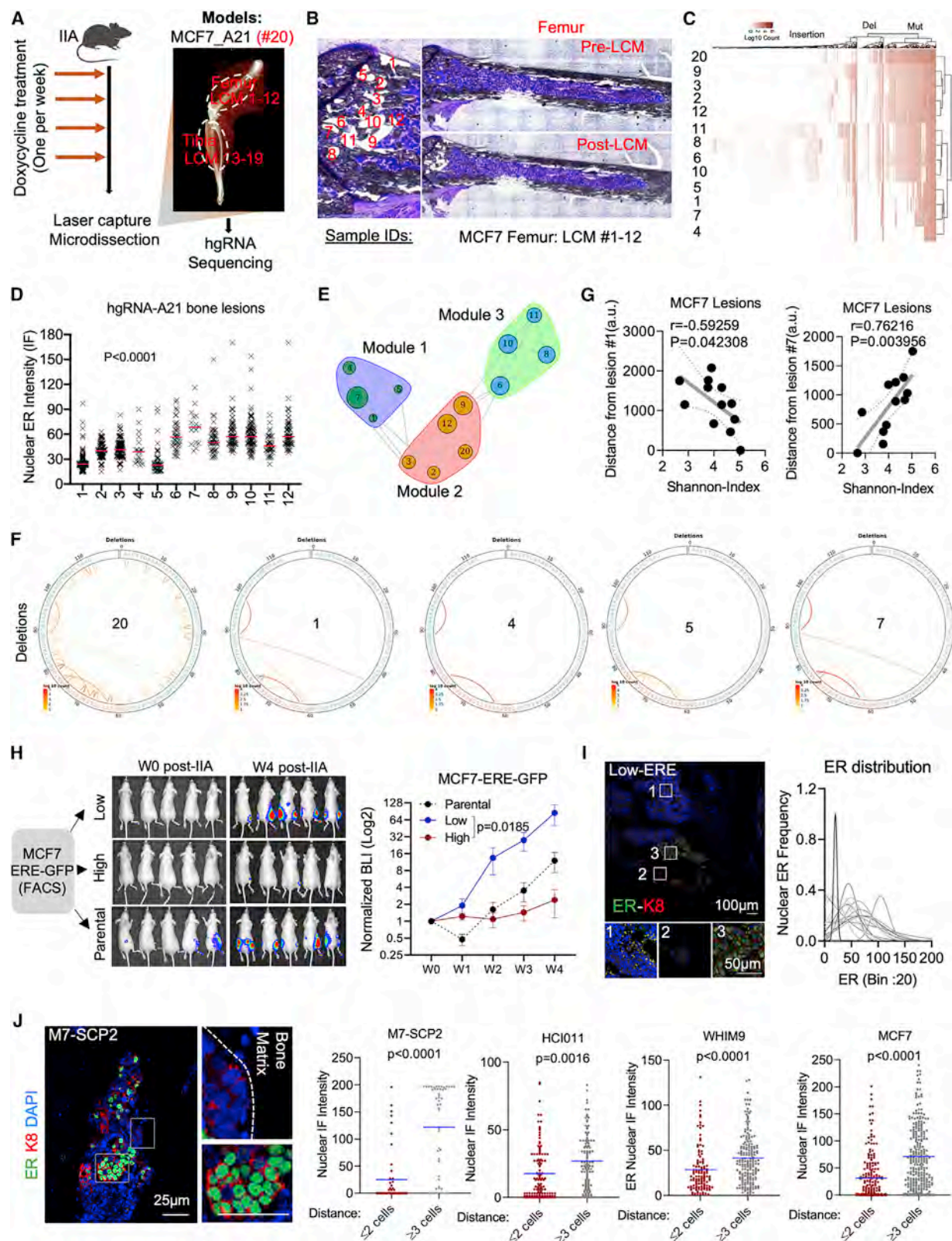
(I) Scheme describing the PET-CT experiment on MCF7 orthotopic or IIA-induced BoM models. 18F-FES and 18F-FDG imaging were performed 48 h apart, at week 1 and 7 post tumor transplantation.

(J) PET/CT scans showing the maximum intensity projection (MIP) of 18F-FDG and 18F-FES in bone as described in I. MCF7 BoMs were generated using IIA injection. Red arrows indicate tumor location. Scale: 0.2–0.5 SUV-bw (week 1) and 100–200 SUV-bw (week 7).

(K) Axial view of PET/CT scans depicting the uptake of 18F-FDG and 18F-FES in small and large lesions of MCF7 orthotopic tumors. Early time point (week 1) and late time point (week 7) were used to depict non-palpable orthotopic tumor stage (small <2 mm) and the palpable tumor stage. Red arrows and scale: as in (J).

(L) Relative quantification of radiolabeled 18F-FES uptake in small and large lesions of orthotopic and BoMs. Each dot represents the mean standard uptake values (mean SUV-bw) of 18F-FES normalized to the mean SUV of 18F-FDG for each mouse. Mann-Whitney U test is used for statistical analysis; $n = 5$ mice per group.

(A–L) p values were computed by two-tailed unpaired Student's t tests unless otherwise noted.



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The evolving barcode system was introduced to MCF-7 cells and was allowed to accumulate mutations before tumor transplantation to the bone by IIA injection (Figure 2A). This created a baseline barcode diversity so that independent clones in the bone can be distinguished. Further evolution after IIA injection helps deduce the parent-child relationship between clones. We used laser-captured micro-dissection (LCM) to isolate cancer cells in different regions of established BoMs (Figures 2B and S2A). DNA purification and PCR-mediated enrichment of hgRNA sequences were performed before sequencing (Figures 2A, 2B, and S2A). The hgRNAs were then used to deduce the phylogenetic relationship among cancer cells in different lesions (Figures 2C and S2B). A consecutive section of the bone was used for immunofluorescence (IF) staining of ER so that the nuclear ER expression of each lesion could be superimposed with the barcodes. ER expression was highly variable across different lesions in the femur (Figures 2D and S2C) and tibia (Figure S2D), indicating an increased phenotypic heterogeneity of the BoM.

In the metaphyseal area of femur, 12 lesions were grouped into three clusters (Figure S2E). Interestingly, ER expression varies within each cluster, which provides strong evidence against genetic traits as a determinant of the ER level in BoM (Figure 2E). Similar observations were found in lesions derived from tibia (Figure S2F). A notable example was the *module 1*, a cluster formed by lesions #1, #4, #5, and #7. The barcodes of these lesions are highly similar (Figures 2F and S2G), indicating close phylogenetic distance. Additionally, these lesions have a distinct spatial distribution with a close proximity to the growth plate (Figure S2H). Lesion #1 exhibited a low ER expression but the highest Shannon entropy among all 12 lesions, whereas lesion #7 was the opposite. Both ER expression and Shannon entropy of other lesions varied as functions of distance to these two extreme lesions (Figure 2G). Considering that the Shannon entropy is correlated with the “age” of cancer cells (Zhang et al., 2021), lesion #1 is likely to be parental to lesions #4, #5, and #7. The fact that their ER levels greatly varies, support our hypothesis that ER[−] lesions may give rise to ER⁺ lesions as BoMs progresses.

The analysis of all lesions (#1–19) derived from the same hind limb revealed surprising similarities between lesions from femur and tibia (Figure S2E). Despite physical barriers, lesion #15 from tibia shared similar mutations with lesions #6, #8, #10, and #11

from femur, suggesting a femur-to-tibia seeding (Figure S2E). This metastasis-to-metastasis seeding is further investigated in our accompanied study (Zhang et al., 2021).

ERE-GFP^{Low} cells drive BoM progression and reconstitute ER heterogeneity

As an independent approach to trace the fate of ER[−] cancer cells in BoM, we introduced a reporter system, namely, GFP expression driven by the estrogen-responsive elements (ERE-GFP). We performed IIA injection of ERE-GFP^{high} and ERE-GFP^{low} cells, respectively. The bone colonization capacity of ERE-GFP^{Low} cells was over 30-fold higher compared with ERE-GFP^{high} cells (Figures 2H and S2I), consistent with the finding that the ER[−] subset of MCF-7 cells enrich stemness (Fillmore et al., 2010). Interestingly, BoMs established by ERE-GFP^{Low} cells exhibited heterogeneous ER expression, similar to those derived from parental cells (Figures 2I and S2J) and lesion sizes were associated with ER expression (Figure S2K). This experiment provides additional support for the conclusion that ER^{Low} cells may generate ER^{high} cells in BME.

BC lesions tend to associate with areas of new bone matrix deposition (Figure S2L). We examined the spatial distribution of ER expression relative to bone matrix. Cancer cells apart from lesion borders were more likely to restore ER expression compared with those at the border (Figure 2J). Thus, ER recovery tends to occur first toward the center of a BoM lesion, leading to the hypothesis that the interactions between metastatic cells and adjacent bone cells drive the transient loss of ER.

Direct interaction with OGs mediates the loss of ER expression

To identify bone cells that causes ER loss in BMMs, we assessed the spatial relationship between ER expression and various bone cells, including osteoclasts (RANK⁺), endothelial cells (CD31⁺), myofibroblasts/bone stromal cells (α SMA⁺), and OGs (ALP⁺). In WHIM9, HClO11, and MCF-7 models, RANK expression exhibited a positive association with nuclear ER intensity at a single-cell level (Figure 3A). In contrast, negative associations were observed for endothelial, fibroblasts, and OGs (Figures 3B and 3C). Among these, the correlation of ALP⁺ OGs is most consistent across different models (Figure 3C), suggesting that OGs may be the cell types driving the loss of ER.

Figure 2. Tracing ER expression in bone metastases by evolving barcodes and ERE-reporter

- (A) Experimental design of BoM lineage tracing using an inducible CRISPR-Cas9 hgRNA evolving barcoding system. iCas9-expressing MCF7 cells were stably transduced with homing guide RNA A21 (hgRNA-A21) before transplantation to bone and induced weekly for 4 weeks before LCM and targeted sequencing. We collected 19 lesions from femur (#1–12) and tibia (#13–19). Barcoded parental cells were labeled as #20.
- (B) Exact map of mouse femur before and after LCM of metastatic lesions #1–12.
- (C) Heatmap showing hierarchical clustering of bone lesions #1–12, based on hgRNA-A21 mutations.
- (D) IF quantification of SCs nuclear ER in lesions #1–12 (femur). (n = 12 lesions); p value: ordinary one-way ANOVA.
- (E) Modular organization of lesions #1–12 following a high-dimensional undirected analysis of barcode mutations. Node sizes represent mean intensity of ER expression.
- (F) Circus plot showing barcode deletions in bone lesions clustered in module 1 (see E). #20: Pre-injected cells.
- (G) Scatter plot showing Pearson correlation (R) between the Shannon index of bone lesions and their relative distance to lesion #1 or #7. p value: two-tailed t test.
- (H) IIA-induced BoM from ERE-GFP reporter MCF7 cells. ERE-GFP^{Low} and ERE-GFP^{high} MCF7 cells were sorted based of their GFP expression. Tumor growth was measured by bioluminescence. (n = 5–8 mice); p value: two-way ANOVA. Error bars: mean $\pm$ SEM.
- (I) IF staining of ER in BoM derived from ERE-GFP^{Low} MCF7 cells. The Gaussianized ER distribution is based on nuclear intensity of SCs; peak: mean ER expression per lesion.
- (J) IF staining depicting a spatial distribution of ER based on the location of M7-SCP2 lesions relatively to the bone matrix. Scale bars: 25 μ m. Dot plots represent nuclear ER expression in cells proximal (≤ 2 cell distance) or distal (≥ 3 distance) to the bone matrix. p values: two-tailed unpaired Student's t tests.

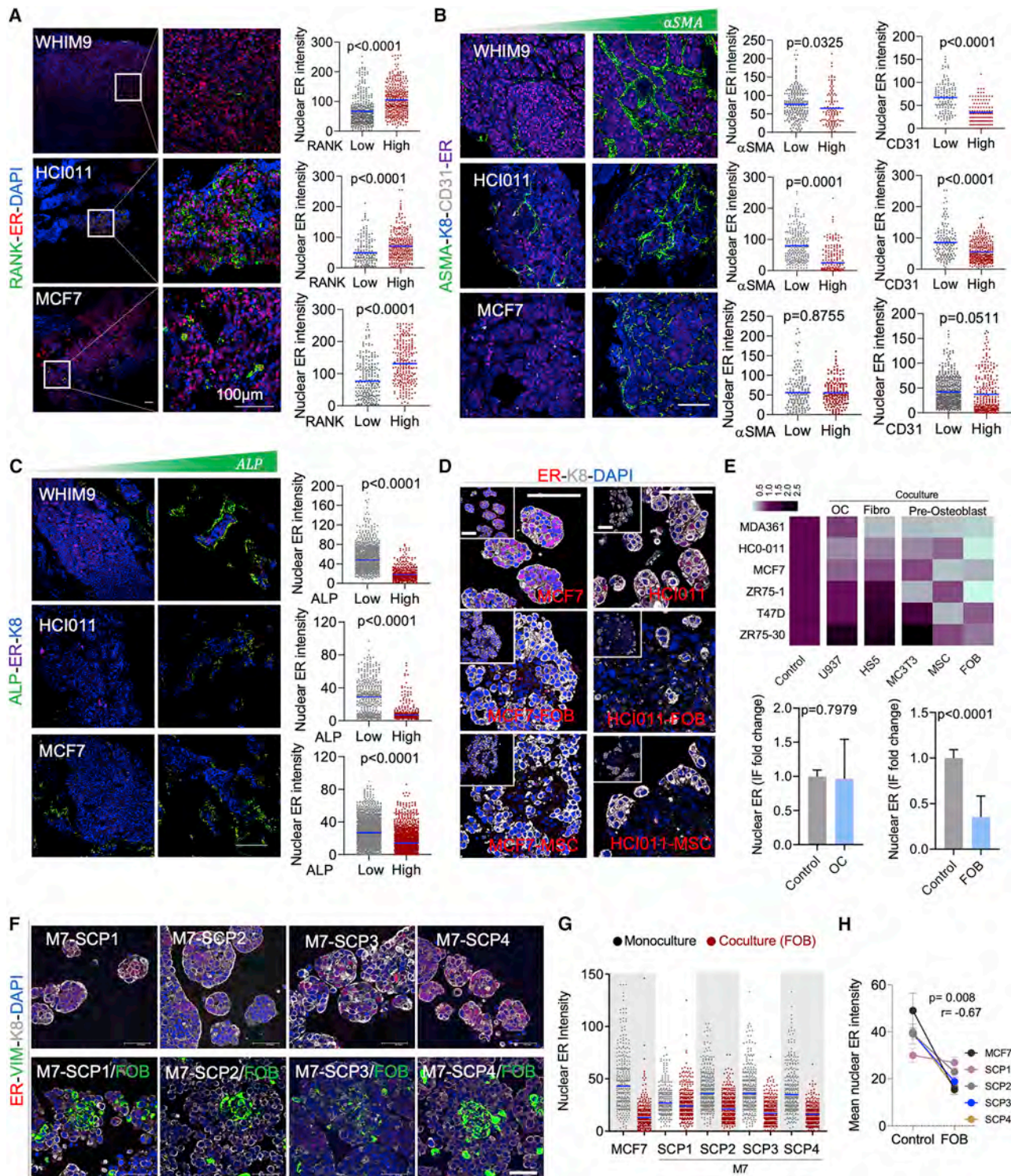


Figure 3. OGs promote the loss of ER expression and reduction of ER activities during early stages of bone colonization

(A) IF of BoMs showing ER expression in PDXs (H1011 and WHIM9) and MCF7 cells, relatively to receptor activator of nuclear factor- κ B (RANK) expression in osteoclasts. Scale bars: 100 μ m. Dot plots show ER quantification in SCs. ($n = 3-5$ lesions).
(B) IF as in (A). ER is co-stained with alpha smooth muscle actin (α SMA) and cluster of differentiation 31 (CD31) to depict endothelial cells. Dot plots show ER quantification in SCs. ($n = 3-5$ lesions).
(C) IF as in (A). ER is co-stained with alkaline phosphatase (ALP). Dot plots show ER quantification in SCs. ($n = 3-5$ lesions).
(D) IF images of H1011-derived primary cells and MCF7 cells in 3D monoculture and co-culture with FOBs and MSCs. Scale bars: 100 μ m.

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To further study this interaction, we employed a fetal OB line (FOB) and a human MSC line to represent OGs. Luminal-like cancer cells and OGs form heterotypic organoids in 3D suspension co-culture, which recapitulated several aspects of cancer-niche interaction (Wang et al., 2015). Co-staining of ER and keratin 8 (K8, a marker of luminal cells) in 3D co-cultures revealed a loss of ER in MCF-7 cell line and HCl011 PDX-derived organoids (Figure 3D) similar to *in vivo* BMMs. MSCs and FOB both induced consistent loss of ER expression across multiple models (Figure 3E). In contrast, U937, a human monocytic cell line that is often used to model osteoclast precursors, did not cause the same changes to the ER expression, supporting the specificity of OGs (Figure 3E).

Importantly, M7-SCPs also exhibited the same alterations upon interacting with FOB (Figures 3F and 3G). In M7-SCP2, -SCP3, and -SCP4, the degree of ER downregulation is comparable with parental MCF-7 cells. In contrast, M7-SCP1 exhibited a lesser decrease (Figures 3G and 3H). Interaction with OGs confers growth advantage on cancer cells, as we previously showed (Wang et al., 2015, 2018). Similar to MCF-7 cells (Figures S3A and S3B), M7-SCP1, 2, and 3 also displayed such an advantage in 3D co-cultures as compared with monocultures. In contrast, the growth of M7-SCP4 was suppressed by FOB (Figure S3C). Thus, different M7-SCPs possess a variable capacity of orthotopic tumor initiation, bone colonization, FOB-mediated growth promotion, and ER downregulation (Figure S3D). This pre-existing heterogeneity supports the importance of clonal selection in metastasis, which has been repeatedly demonstrated in previous studies (Bos et al., 2009; Kang et al., 2003; Minn et al., 2005). However, the BME-induced, adaptive change is less appreciated (Figures 3F–3H).

Hyperactive ER activities can lead to ER protein degradation (Nawaz et al., 1999). Therefore, decreased ER expression could paradoxically suggest an enhanced ER signaling. Upon co-culturing with FOB, cancer cells reduced the transcription of ER mRNA (Figure 4A), as well as ER transcription activities as indicated by a luciferase reporter driven by a promoter containing ER-responsive elements (ERE-luciferase) (Figure 4B). The ER target progesterone (PR) was also downregulated in BMMs (Figure 4C). These data indicate that the transient loss of ER is not a reflection of hyperactive ER signaling, but rather the cause of decreased ER signaling.

Interaction with OGs in the BME leads to resistance to endocrine therapies

Downregulation of ER may impact endocrine therapies. We examined the effects of fulvestrant, tamoxifen, and estradiol on ER⁺ cancer cells with or without co-culture of FOB. The presence of FOB diminished the effects of these agents on ER nuclear localization (Figure 4D) and blunted the anti-proliferative effects

of tamoxifen and fulvestrant (Figure 4E). The same results were observed using M7-SCPs (Figure 4F), indicating a process independent of genetic selection. We next examined the reversibility of “bone-entrained” effects by inoculating M7-SCP2 cells into bone via IIA. Cancer cells were retrieved after establishment of BMMs, resulting in “bone-entrained” M7-SCP2 cells (M7-SCP2-Bo) (Figure S4A). M7-SCP2-Bo cells remained resistant to fulvestrant in early passages, but this resistance diminished as cells were expanded in cultures (Figure 4G), suggesting that BME-induced phenotypic shift is not stably inherited.

We next examined responses of ER⁺ cancer cells to estrogen deprivation in BME (Figure S4B). Combined ovariectomy and letrozole treatment could significantly impede orthotopic tumor growth in both MCF-7 and ZR75-1 models (Figures S4C and S4D) but failed to reduce BoM colonization at early time points (Figures 4H–4M). The response to estrogen deprivation was partially recovered in MCF-7 cells after week 3 (Figures 4H–4J), further supporting that the resistance may be reversible as metastases further progress. However, ZR75-1 bone lesions remained resistant at later time points (Figures 4K–4M). Hence, not all BoMs restore endocrine sensitivity. In ZR75-1, despite the partial reversion of ER expression in macrometastases, PR expression remained repressed (Figures S4E and S4F). Thus, downstream ER signaling may not recover together with ER expression during BoM progression.

Downregulation of ER in BoM is partially mediated by direct cell-cell contact and gap junctions

We previously reported that heterotypic gap junctions between cancer cells and OGs mediate calcium influx to the former and activates calcium signaling (Wang et al., 2018). We asked if the gap junction and calcium signaling may mediate ER downregulation and endocrine resistance. Suppression of gap junction by a peptide, GAP19, or calcium signaling by a small molecule inhibitor, FK506, partially restored ER expression in co-cultures with FOBs (Figure S5A). This effect was small but noticeable and was further supported by a converse experiment showing that high [Ca²⁺] in the medium decreased ER expression (Figure S5B). Inhibition of calcium signaling also reduced the growth advantage conferred by FOB (Figure S5C), and enhanced endocrine therapies in bone-in-culture array (BICA) (Figure S5D), which is an *ex vivo* platform that recapitulate BME and cancer-niche interactions (Wang et al., 2017). Taken together, gap junction and calcium signaling contribute to inhibit ER expression in BMMs.

Unbiased profiling uncovered global phenotypic shift of ER⁺ cancer cells

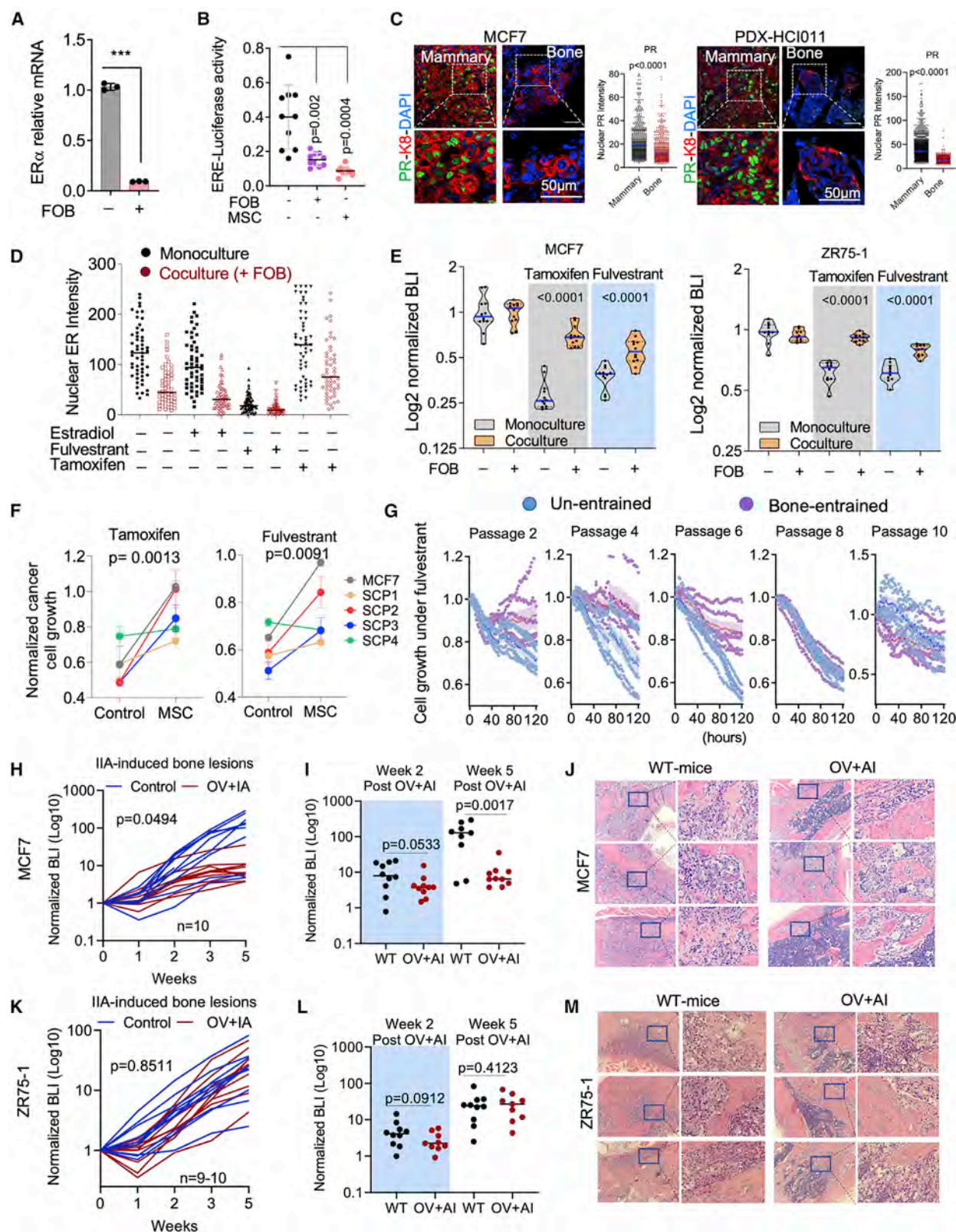
We used multiple approaches to discover additional mechanisms underpinning BME-induced ER downregulation (Figure 5A). Translating ribosome affinity purification followed by

(E) Heatmap showing the mean intensity of ER in primary cells (HCl011) and BC cell lines (MDA-MB-361, MCF7, ZR75-1, T47D, ZR75-30) in 3D monoculture (control) or co-culture with osteoclast precursors (U937), bone marrow stromal cells (Hs5), mouse pre-osteoblasts (MC3T3), human mesenchymal stem cells (MSC) and human pre-osteoblast (FOB). Histogram shows ER expression in monoculture versus co-culture of multiple cell lines with U937 or FOB cells; (n = 3 biological replicates). Error bars: mean ± SD.

(F) IF showing ER expression in M7-SCPs in 3D monoculture or co-culture with FOB cells. Vimentin (VIM). Scale bars: 50 μm.

(G) IF quantification of ER expression in M7-SCP1 to 4; (n = 3 biological replicates).

(H) Graph representing ER expression in cancer cells alone or in co-cultured with FOB cells. Spearman correlation (R); (n = 5 cell lines); Error bars: mean ± SEM. (A–H) p values were computed by two-tailed unpaired Student t tests unless otherwise noted.



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RNA-seq (TRAP-seq) was applied to profile transcriptome in cancer cells interacting with OGs in 3D suspension co-cultures without dissociating the two cell types. TRAP-seq revealed that MSCs diminished the impact of endocrine perturbations on ER⁺ cancer cells (Figure S5E). We also validated that GJA1, the gene encoding connexin 43, was upregulated by MSCs in co-cultures and exhibited a strong inverse correlation with ER expression (Figure S5F), further indicating a role of gap junctions in downregulating ER. However, the conditioned medium of OGs also causes ER downregulation and endocrine resistance (Figure S5G), indicating paracrine mechanisms.

According to TRAP-seq, over 1,100 genes are significantly increased by MSC co-cultures (FDR <0.05 and fold change >2), which indicates a global phenotypic alteration. Using PAM50 signatures, we observed a shift from luminal to basal subtype (Figure 5B). Several pathways changed significantly in MSC-interacting cancer cells, including the decrease in ER signaling and increase in epithelial to mesenchymal transition (EMT) and STAT3 signaling (Figure 5B), all of which indicated dedifferentiation and stem-like activities (Mani et al., 2008; Martotta et al., 2011; Pfefferle et al., 2015). The PANTHER classification system identified a number of pathways overrepresented in the altered genes, including several related to epigenomic regulation of gene expression (e.g., PRC2), stemness-related pathways (e.g., WNT and Notch), and receptor tyrosine kinase (RTK) signaling (Figures 5C and S5H). Some of these pathways have been previously implicated in BoM and therapeutic resistance (Andrade et al., 2017; Esposito et al., 2019; Sethi et al., 2011; Zheng et al., 2017). These findings indicate that OGs induce an epigenomic landscape alteration in ER⁺ BC cells toward more ER-independent and stem-like states.

Reverse phase protein array (RPPA) was used to profile over 236 key proteins and phospho-proteins in cancer cells that have been extracted from BME. Comparisons were made between MCF-7 and MCF-7-Bo and between M7-SCP2 and M7-SCP2-Bo. We identified the proteins and phospho-proteins

that are significantly altered (Figures S5I and S5J). The bone-entrained cells exhibited reduced ER signaling (Figure 5D), enhanced stemness (Figure 5D), increased mesenchymal markers (Figure 5E), and increased RTK expression (Figure 5F). The most upregulated protein in both MCF-7-Bo and M7-SCP2-Bo was PDGFR β (Figures S5J and S5J). Overall, these changes revealed a global phenotypic shift toward a more dedifferentiated status (Ginestier et al., 2007; Guo et al., 2012; Mani et al., 2008; Tam et al., 2013; Ithimakin et al., 2013). One notable osteogenic cell-induced change is the acquisition of a hybrid EMT status (Figure 5E). We performed co-IF staining of epithelial markers (E-cadherin and cytokeratin 8) and a mesenchymal marker (Vimentin). A small proportion of double-positive cells were observed, usually located at the border between metastases and bone matrix, where OGs are located (Figure 5G). Thus, the interaction with OGs does not simply cause EMT, but rather induce phenotypic plasticity and confer stemness.

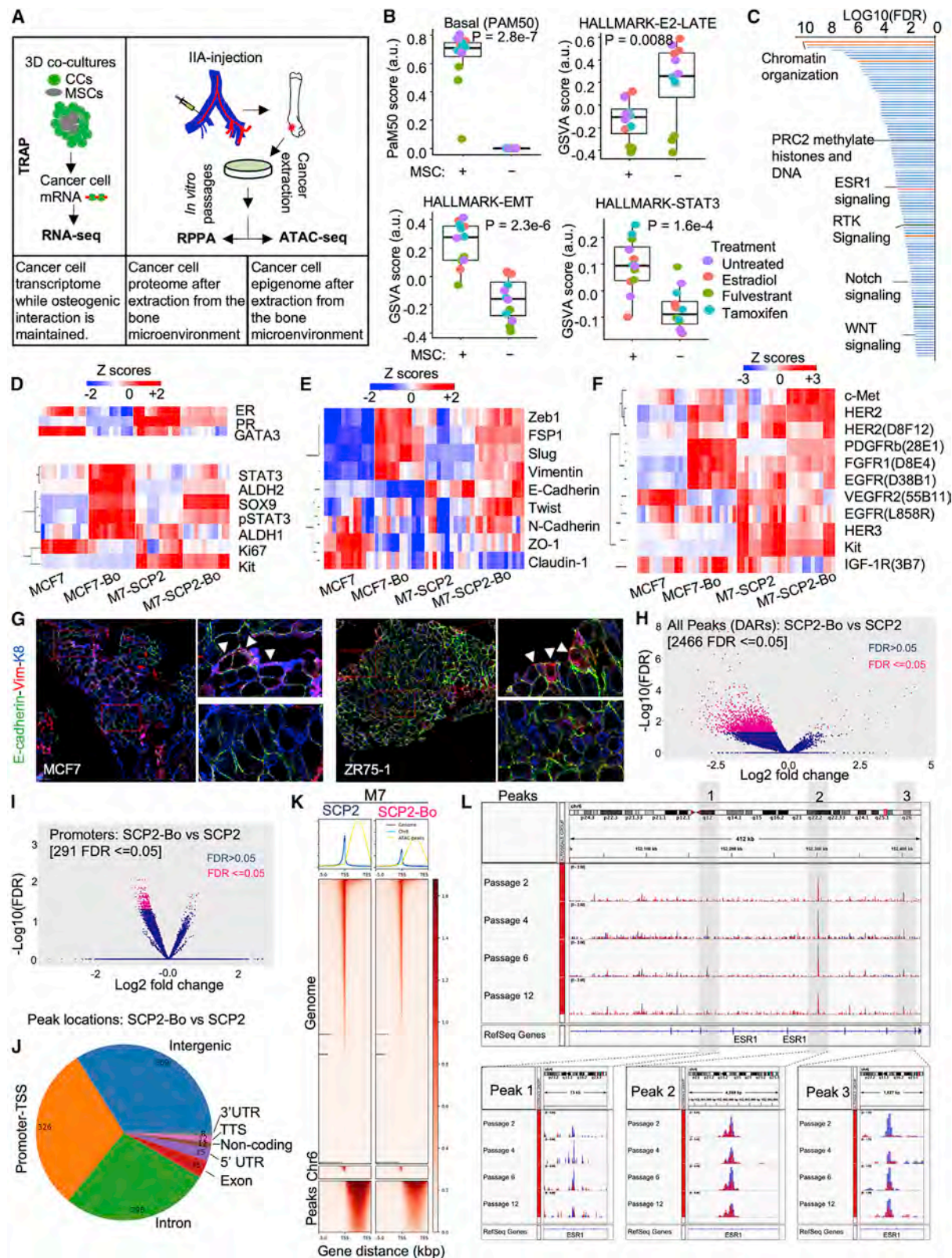
Assay for transposase-accessible chromatin using sequencing (ATAC-seq) (Buenrostro et al., 2015) was used to evaluate epigenetic changes occurring at the chromatin level of cancer cells extracted from bone. The differentially accessible regions (DAR) between parental and bone-entrained cells were decreased in the latter in general (Figures 5H–5J), indicating a global shift toward heterochromatin formation. This was further supported by a global decrease in chromatin accessibility (Figure 5K). We identified two major peaks around ESR1 gene with reduced chromatin accessible in bone-entrained cells (Figure 5L) (Peaks 1 and 3). The reduction reversed over multiple passages *in vitro* (Figures 5L and S5K). Overall, these results indicate a reversible epigenetic reprogramming in cancer cells interacting with BME.

FGFR and PDGFR pathways contribute to phenotypic changes in BMMs

Among all pathways altered in BME, PDGFR β and FGFR1 pathways are of particular interest. PDGFR β exhibited the highest

Figure 4. OGs confer endocrine resistance

- (A) Relative mRNA expression of ESR1 in 3D monoculture or co-culture of MCF7 with FOB. Data result from FACS-sorted MCF7. (n = 3 technical replicates). Error bars: mean $\pm$ SD.
- (B) Dot plot representing ER transcriptional activity in MCF7 cells expressing PGL2 ERE-luciferase reporter. MCF7 cells were cultured in 3D with or without OGs (FOB and MSC) for 7 days. (n = 10 technical replicates); Error bars: mean $\pm$ SD.
- (C) Confocal images showing the expression of progesterone receptor (PR) in IIA-induced BoM from MCF7 and HCl011. Dot plots show nuclear PR IF intensity. (n = 3 lesions).
- (D) Dot plots depicting ER intensity in 3D mono- and co-culture of MCF7 cells with FOB following 24 h treatment with 10 nM 17 β -estradiol, 20 nM fulvestrant, and 100 nM 4-hydroxytamoxifen (tamoxifen); n = 5 fields.
- (E) Violin plot showing the response of luciferase-labeled MCF7 and ZR75-1 cells to 100 nM of 4-hydroxytamoxifen (tamoxifen) and 20 nM of fulvestrant in 3D mono- or co-culture with OGs (FOB). Bioluminescence was acquired 72 h post-treatment. (n = 12 and 10 technical replicates for and ZR75-1).
- (F) Graphs representing the proliferation of MCF7 cells and M7-SCP1-4 in monoculture and MSC co-culture following 1 week of treatment with 20 nM fulvestrant or 100 nM tamoxifen; n = 5 cell lines. Error bars: mean $\pm$ SEM.
- (G) Time course experiment depicting growth kinetics of un-entrained and bone-entrained M7-SCP2 cells in vehicle or 20 nM fulvestrant conditions; n = 6 technical replicates.
- (H) Growth curve showing response of IIA-induced MCF7 BoMs to estrogen depletion. Ovariectomized (OV) mice were additionally treated with letrozole (OV+AI), daily. Results are based on BLI. (n = 10 mice); p value: two-way ANOVA.
- (I) Dot plot showing BoM growth in wild-type (WT) and OV+AI mice at week 2 and week 5 post tumor transplantation; n = 10 mice. p value: two-tailed unpaired Student's t test.
- (J) H&E staining showing MCF7 metastatic lesions in wild-type (WT) and "OV+AI" groups.
- (K) Growth curve depicting the response of ZR75-1-derived BoM as in (H). (n=9–10 mice); p value: two-way ANOVA.
- (L) Dot plot showing statistical growth differences in ZR75-1 as in (I).
- (M) H&E staining of ZR75-1 metastatic lesions as in (J).
- (A–M) p values were computed by two-tailed unpaired Student's t tests unless otherwise noted.



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fold change in both models (Figures S5I and 5J) and was shown to determine the subtype of BC and mediate cancer stem cell activities (Lehmann et al., 2011; Tam et al., 2013). Multiple FGF ligands and receptors were upregulated in human BoMs compared with matched primary tumors (Priedigkeit et al., 2017). FGF signaling regulates stem cell compartment in ER⁺ BC (Fillmore et al., 2010). Using a literature-based network analysis platform (<https://string-db.org/>) (von Mering et al., 2005), we found that FGF2 connects ER, FGFR1, and PDGFRB (Figure 6A), suggesting a pivotal role of FGF2 in ER downregulation and endocrine resistance.

FGF2 is the highest expressed FGF ligands by FOB cells (Figure 6B). IF staining of FGF2 in BMMs revealed a positive correlation with ALP⁺ OGs (Figures 6C and S6A) and a negative correlation with nuclear intensity of ER (Figures 6D and 6E). Functionally, recombinant FGF2 treatment decreased ER expression in multiple cell lines including M7-SCPs (Figure 6F), and induced resistance to fulvestrant (Figure 6G). Conversely, a potent FGFR inhibitor, BGJ398, reversed the fulvestrant resistance of ER⁺ cancer cells in BICA (Figure 6H).

PDGFRB is highly expressed in the bone-entrained cells at both protein and mRNA levels (Figure S6B). Direct interaction between cancer cells and OGs was required for PDGFRB upregulation (Figure S6C). Among all PDGF ligands, PDGF-DD, but not PDGF-BB or PDGF-CC, significantly promoted the therapeutic resistance (Figures S6D and S6E), while suppressing the ER expression (Figures S6F and S6G). Like FGFR, the inhibition of PDGFR signaling by sunitinib partially abolished the cancer-promoting effects of FOB cells in 3D co-cultures (Figure S6H), further supporting the important roles of both FGFR and PDGFR signaling in the BME-induced endocrine resistance.

The complicated impact of BME converges on an EZH2

We next asked how the discovered pathways cooperate to silence ER and increase phenotypic plasticity. In the Epigenomic Roadmap database, FGF2-regulated genes are enriched with tri-methylation of H3K27, and sensitive to perturbation of EZH2 (Figure 6I). Consistently, PRC2 methyltransferase activity is enhanced in cancer cells co-cultured with MSCs (Figure 5G). Treatment of recombinant FGF2 increased H3K27me3 and

EZH2 (Figures 6J, 6K, and S6I) but did not affect other H3 modifications (Figure S6J). Conversely, treatment of BGJ398 decreased EZH2 expression in 3D cancer-MSC co-cultures (Figure 6J). PDGF-DD yielded similar effects (Figures S6I and S6J). Furthermore, calcium signaling affected EZH2 expression at the RNA level (Figure S6K). Thus, the pathways that downregulate ER seem to converge on the regulation of EZH2.

EZH2 is a reliable marker for cancer stemness (Kim and Roberts, 2016; Zhou et al., 2002). In ER⁺ cancer cells, the PRC2 target genes were concertedly downregulated, whereas a stemness signature (Varambally et al., 2020) was upregulated, by co-culturing of OGs (Figures S6L–S6N), validating the connection of EZH2 to cancer stemness. In addition, cancer-cell-intrinsic EZH2 expression was specifically increased in BME compared with other organs (Figure 6L).

EZH2 has been shown to silence ER expression in previous studies (Reijm et al., 2011). To validate this, we carried out IF staining in 3D co-cultures and observed an inverse correlation between EZH2 and ER expression both in 3D cultures and in bone lesions at a single-cell level (Figures 6M and 6N). Inhibition of EZH2 enzymatic activity by an EZH2 inhibitor (EPZ01989) (Campbell et al., 2015) led to restoration of ER expression at the RNA level (Figure 6O). The inverse changes of ER and EZH2 were also observed *in vivo* between BMMs and macrometastases (Figure 6P). Finally, the BME-induced increase in H3K27me3 was reversible after several passages *in vitro*, again suggesting a transient impact (Figure 6Q).

EZH2 inhibition induced ER expression in a murine model, which is abolished by OGs in bone lesions

We sought to validate our findings in immunocompetent hosts. Most murine BC cell lines are ER[−], thereby limiting the possibility of syngeneic experiments (Derose et al., 2011). However, some murine models express ER in early-stage tumor progression, e.g., MMTV-PyMT (Lin et al., 2003; Medina et al., 2002). AT3 is a cell line derived from MMTV-PyMT (Guy et al., 1992). We reasoned that it might express ER at some stage and examined if EZH2 inhibition restores ER expression in AT3. In mammary tumors, the transient treatment of EZH2 inhibitor increased ER expression in a durable fashion and to a level exceeding the threshold defining

Figure 5. BME drives a global phenotypic shift involving multiple pathways

- (A) Experimental summary diagram to evaluate molecular in cancer cells exposed to BME. TRAP-seq was performed on 3D co-culture of MCF7 and OGs (MSCs), RPPA on un-entrained (MCF7 and M7-SCP2) and bone-entrained cells (MCF7-Bo and M7-SCP2-Bo), and ATAC-seq on un-entrained (M7-SCP2) or bone-entrained (M7-SCP2-Bo) cells.
- (B) Box plot depicting gene signature alternations in MCF7 monoculture (MSC-) and co-cultures (MSC) from TRAP-seq. Analysis was performed using a non-parametric and unsupervised GSVA (Hänzelmann et al., 2013). Specific colors represent different treatment conditions. (n = 4 biological replicates each with 3 technical replicates); p value: two-tailed unpaired Student's t test.
- (C) Waterfall plot showing the gene ontology analysis of TRAP-seq data from PANTHER classification system, based on false discovery rate (FDR).
- (D) Heatmap depicting expression changes in luminal and stemness-related markers from RPPA data. Parental cells (MCF7 and M7-SCP2), and bone-entrained BC cells (MCF7-Bo and M7-SCP2-Bo) are compared; N: 4 biological replicates and 3 technical replicates.
- (E) Heatmap depicting EMT/MET markers from RPPA data as described in (D).
- (F) Heatmap depicting receptor tyrosine kinases from RPPA data as described in (D).
- (G) IF showing vimentin, E-cadherin, and keratin 8 expression in IIA-induced bone MCF7 and ZR75-1 BoMs.
- (H) Volcano plot showing epigenetic reprogramming of bone-entrained M7-SCP2 cells based on differentially enriched peaks from ATAC sequencing analysis. 2,644 peaks were significantly altered in bone-entrained M7-SCP2 (FDR ≤ 0.05) and highlighted in pink.
- (I) Volcano plot based on opened promoters identified by ATAC-seq analysis. FDR < 0.05 is highlighted in pink.
- (J) Pie chart depicting the genomic distribution of differentially altered peaks between un-entrained and bone-entrained M7-SCP2 cells.
- (K) Heatmaps and summary plots showing chromatin opening near the transcription start site TSS. 5,000 bp before and after TSS are represented.
- (L) Genomic track showing peak variations in the ESR1 gene of un-entrained (M7-SCP2) and bone-derived (M7-SCP2-Bo) cells in blue and red color, respectively. 3 major peaks are highlighted epigenetic reversibility in M7-SCP2-Bo *in vitro*.

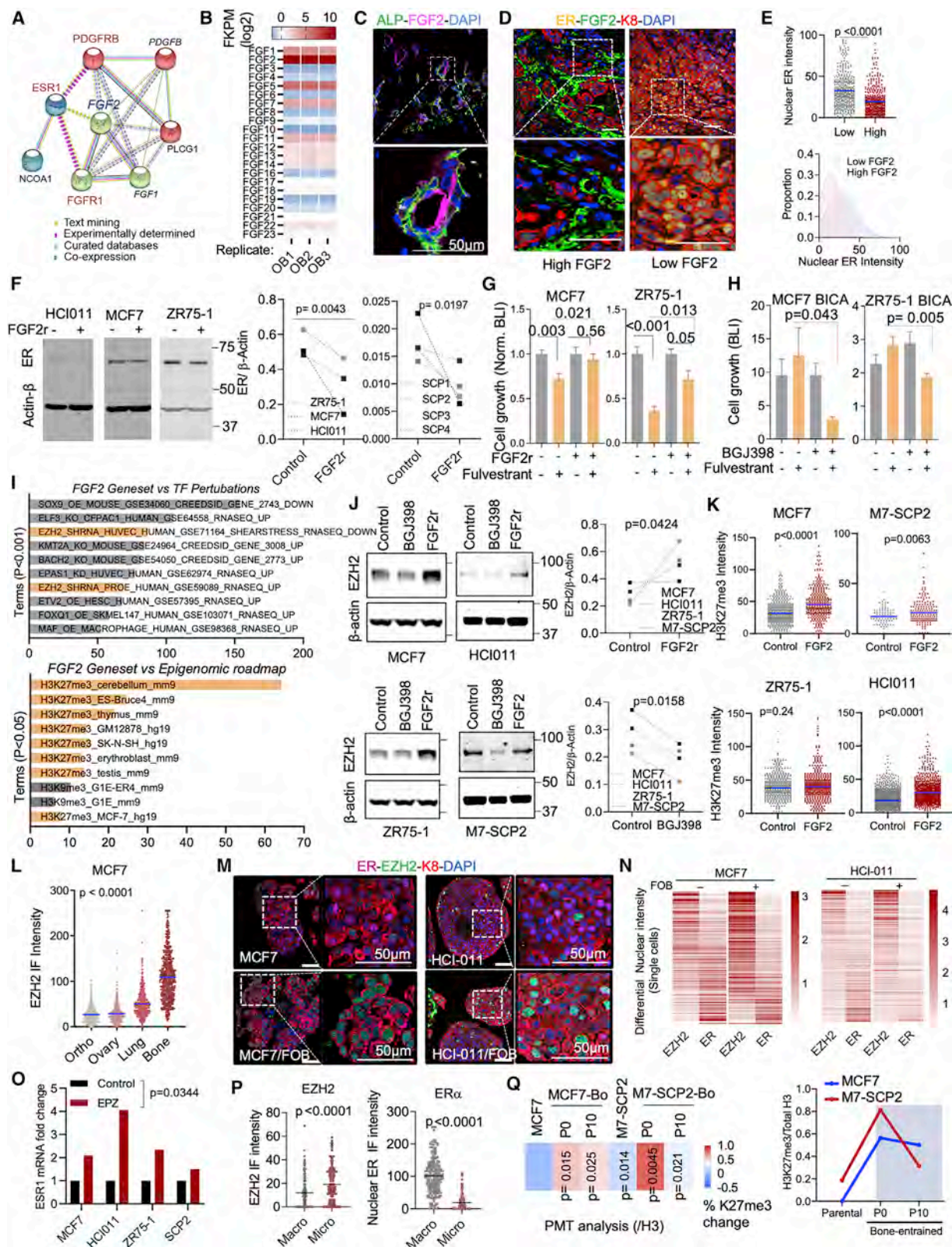


Figure 6. Osteogenic cell-secreted FGFs promote endocrine resistance

(A) Network depicting functional protein association between FGFR1, PDGFRB, and ER using the STRING database. *K* means clustering ($k = 3$) was used to represent 3 major centroids (depicted as red, green, and cyan spheres) and their most closely associated proteins based on unsupervised data mining.

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ER⁺ tumors (Figures 7A, 7B, and S7A). Although the re-expression of ER could not restore downstream estrogen signaling, it recapitulated upstream regulation of ER expression by EZH2. Therefore, we went on to determine how the aberrant expression of ER in AT3 cells might respond to BME. After IIA injection, ER expression in AT3 was lost again in syngeneic mice, and this loss was especially pronounced in regions adjacent to bone matrix (Figures 7C and 7D). Importantly, inducible depletion of osterix-expressing osteoprecursor cells abolished the loss of ER (Figures 7C and 7D). Taken together, these data validated that the OGs suppress ER expression in BME.

Short-term inhibition of EZH2 restores sensitivity of BMMs to endocrine therapies

Since EZH2 mediates the BME-induced endocrine resistance, we hypothesized that inhibition of EZH2 should reverse this resistance and synergize with endocrine therapies. Using the EZH2 inhibitor EPZ011989, we confirmed our hypothesis *in vitro* using MCF-7 cells. The synergy is especially strong on MCF-7-Bo cells (Figure S7B). Next, a four-arm *in vivo* experiment was used to ask if combinatory treatment of EPZ011989 and fulvestrant at the microscopic metastasis stage (to mimic adjuvant therapy) could lead to decreased bone colonization. EPZ011989 and fulvestrant had little-to-modest effects when used as single agents. However, the combined treatment inhibited bone colonization and rendered 50% of mice tumor free on the basis of bioluminescence signals (Figure 7E) and microCT (Figures 7F, S7C, and S7D). This is a remarkable effect considering that EPZ011989 treatment only lasts for 3 weeks. EPZ011989 treatment also sensitized ZR75-1 bone lesions to fulvestrant treatment (Figure 7G). Finally, we tested

the combinatory treatment on PDX-based spontaneous BoM models using PET imaging. Pretreatment of mice with EPZ011989 inhibited spontaneous metastasis to bone, as shown in the reduced 18F-FDG uptake (Figures 7H and S7E).

DISCUSSION

Phenotypic plasticity has been recognized as a major driving force of normal development, tumor initiation, and tumor progression (Dravis et al., 2018; Gupta et al., 2019; Lambert et al., 2017). The heterogeneity of ER expression in ER⁺ tumors has long been noticed and may reflect such plasticity. In this study, our data demonstrate that even genetically identical ER⁺ cancer cells exhibit a substantial level of variation in ER expression. We uncovered that the OGs trigger an adaptive epigenomic change in ER⁺ metastatic seeds through both paracrine signaling and direct cell-cell contact and leads to increased phenotypic plasticity and therapeutic resistance. These changes form a transient and reversible effect on cancer cells, including those that are genetically homogeneous, which distinguishes this process from the clonal selection investigated in the past (Bos et al., 2009; Kang et al., 2003; Minn et al., 2005). Indeed, our data suggest a coordinated action between epigenomic adaptation and genetic selection.

Our study identified a number of pathways that are altered in cancer cells by BME. Among these pathways, EZH2-mediated epigenomic reprogramming is a leading candidate for therapeutic intervention. It integrates multiple signals from OGs (e.g., FGF2 and PDGF-DD), and in turn, broadly impacts several downstream pathways related to cancer stemness and metastasis (e.g., WNT and Notch) (Gonzalez et al., 2014; Shi et al.,

(B) Graph showing the expression of all human 22 fibroblast growth factor (FGF) family proteins (FGF1-23) in OGs (FOB) RNA sequencing dataset (n = 3 technical triplicates).

(C) IF images of alkaline phosphatase (ALP) and basic fibroblast growth factor (FGF2/bFGF) in normal bone tissue. Nuclei is shown in blue (DAPI). Scale bar: 50 μ m.

(D) IF images showing decreased ER expression in tumors established in FGF2 enriched BME.

(E) The scatter dot plot represents ER quantification from tumors according to FGF2 enrichment (Low and High) in adjacent stromal cells (n = 3–4 lesions). Mean expression is represented in blue. The Gaussian curve simulates ER distribution based on nuclear intensity. Peaks: mean expression of ER.

(F) Immunoblots depicting the effect of recombinant FGF2 (20ng/ml) on ER expression in multiple BC models including PDX HCl011. Cells were treated for 24 h. ER expression was summarized as connected dot plot graphs; n = 3 and 4 cell lines. p values: one-tailed unpaired Student's t tests.

(G) Histogram showing effect of recombinant FGF2 (20ng/ml) on MCF7 and ZR75-1 cell growth in 3D; n = 6 technical replicates.

(H) Bone-in-culture-array (BICA) assay showing synergistic effects between 2.5 μ M pan FGFR inhibitor (BGJ398) and 20 nM fulvestrant in MCF7 and ZR75-1 models. n = 6 technical replicates.

(I) Annotated bar plot showing the association of histone modifications with basic FGF (FGF2) gene signatures using the Enrichr platform (<https://amp.pharm.mssm.edu/Enrichr/>). Processed ChIP-seq data were obtained from Epigenomic Roadmap Project (Roadmap Epigenomics Consortium et al., 2015). Histograms represent the association score with FGF2 signaling. Signatures are sorted based on p value ranking. Only p values < 0.05 and < 0.01 were shown for the top and bottom panel, respectively.

(J) Immunoblotting showing alteration of EZH2 expression in multiple cells following a 24 h treatment with 1 μ M pan FGFR inhibitor (BGJ398) or 20 nM FGF2 recombinant (FGF2r). Primary cells generated from HCl011 (ER⁺ PDX) were cultured in 3D and treated with 1 μ M pan FGFR inhibitor (BGJ398) or vehicle for 24 h. Normalized EZH2 expression (β -actin) was shown as dot plots. (n = 4 cell lines); p value: one-tailed unpaired Student's t test.

(K) Dot plots depicting the effect of 20 nM recombinant FGF2 on H3k27me3 of multiple ER⁺ BC 3D models, based on quantified IF images (n = 3 technical replicates). p values: one-tailed paired Student's t tests.

(L) IF quantification of EZH2 expression in paired metastases and orthotopic tumor. MCF7 cells were transplanted to bone and to mammary gland of nude mice, which led to tumor formation at multiples sites, including lung, ovary, bone, and mammary gland. Metastatic tissues were harvested for IF quantification and shown as a dot plot graph. (n = 3 images); Error bars: mean $\pm$ SD.

(M) IF showing co-expression of ER and EZH2 in 3D monocultures and co-cultures (+FOB) models of MCF7 and PDX HCl011. Scale bars: 50 μ m.

(N) Heatmap showing relative expression of nuclear ER and EZH2 in MCF7 and PDX HCl011 SCs as depicted in (M).

(O) Effect of the EZH2 inhibitor EPZ011989 on ESR1 mRNA expression after 24 h of treatment; n = 4 cell lines.

(P) Dot plots showing IF quantification of EZH2 and ER in MCF7 BMMs and macrometastases (n = 3–5 lesions). Error bars: mean $\pm$ SD.

(Q) Reversibility of epigenetic changes based on post-translational modification (PTM) analysis. The percent changes in H3k27me3 between parental cells (MCF7), M7-SCP2 and bone-derived (MCF7-Bo and M7-SCP2-Bo) after multiple passages *in vitro* (Passages: P0 and P10). (n = 4 biological replicates); p values are relatively to parental cells. The right panel depicts the temporal epigenetic changes (H3k27me3/H3).

(A–Q) p values were computed by two-tailed unpaired Student's t tests unless otherwise noted.

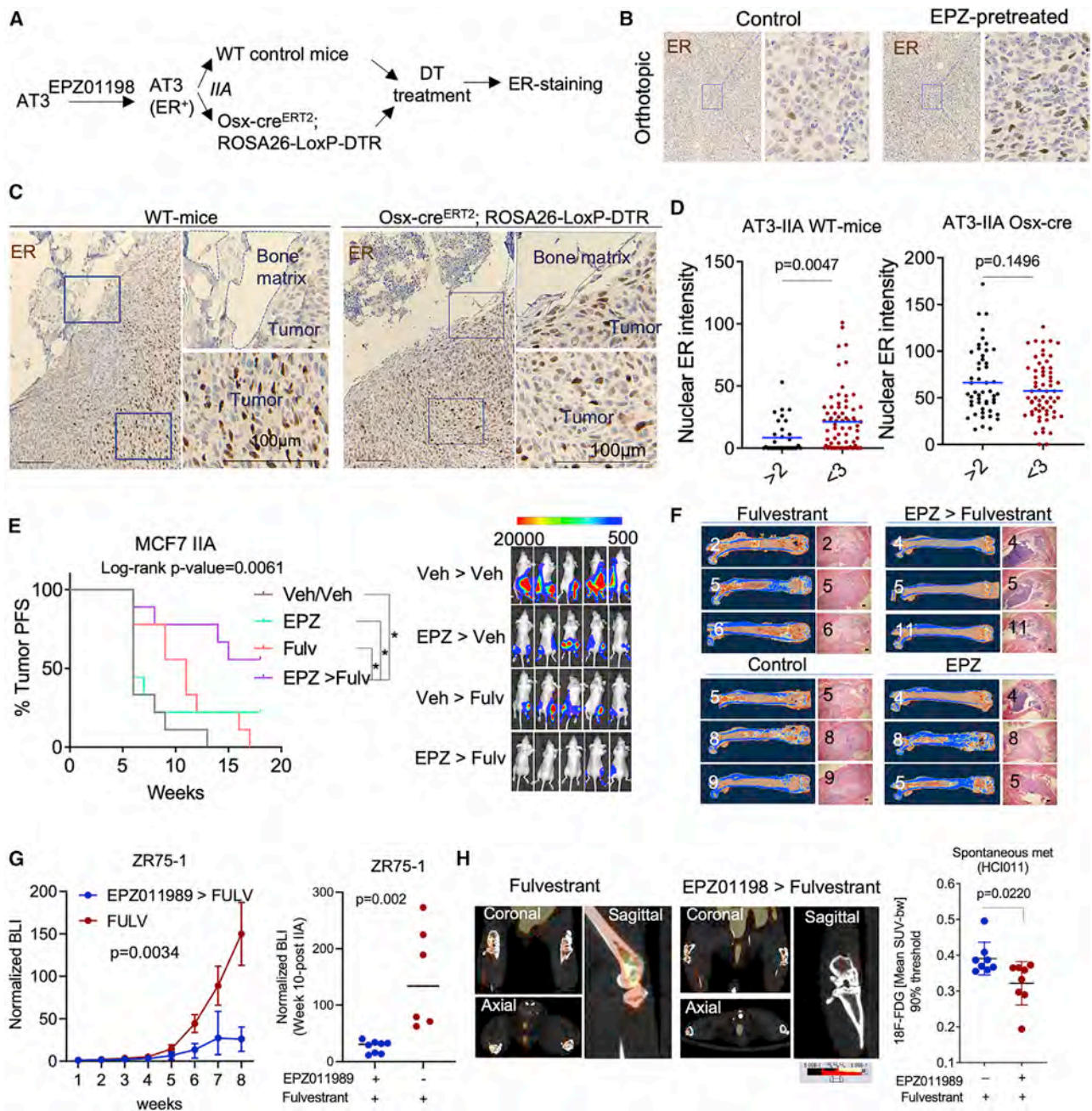


Figure 7. EZH2 integrates multiple signals from BME and drives the phenotypic shift of ER⁺ breast cancer cells

(A) Scheme to evaluate ER loss in syngeneic murine models. AT3 cells were pretreated with EZH2 inhibitor (EPZ01198) for 2 weeks before being transplanted to bone of wild-type or osterix-depleted C57BL/6 mice (Osx-cre^{ERT2}; ROSA26-LoxP-DTR).
 (B) IHC staining depicting ER expression in orthotopic tumors derived from EPZ01198 pretreated AT3 cells.
 (C) IHC staining of ER in BoM models presented in (A).
 (D) Dot plots showing ER expression in IIA-induced AT3 BoM in control (WT-mice) and osteoprogenitor-depleted (Osx-cre^{ERT2}; ROSA26-LoxP-DTR) mice at the SC level.
 (E) Progression free survival (PFS) curve of IIA-induced BoMs following treatment with the EZH2 inhibitor EPZ01198 or the ER inhibitor fulvestrant in combination or as single agents. BL images show the effect of combination treatment on IIA-induced BoMs. (n = 9–10 mice); Log-rank test was used for survival analysis. *p < 0.05.
 (F) microCT and H&E images depicting tumor burden after pre-clinical experiment described in E. All groups revealed BoM formation except for combination treatment group (EPZ > Fulvestrant).
 (G) Growth curve showing the effect of EPZ01198 pretreatment on the fulvestrant response of endocrine resistant ZR75-1 BoMs. Single agent and combination treatment groups are shown in blue and red, respectively. Multiple ANOVA was used for statistical analysis. BLI of metastatic burden at week 8 was shown as dot plot. (n = 6 and 8 mice for fulvestrant and combination group, respectively). Error bars: mean ± SD.

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2007). Moreover, potent and selective EZH2 inhibitors are available and are being clinically investigated in other diseases (Italiano et al., 2018), making it relatively easy for future clinical applications. Pharmacological inhibition of EZH2 promotes a global landscape change of histone marks (Huang et al., 2018). Tumors that developed resistance to histone demethylase KDM5A/B had increased EZH2 expression (Hinohara et al., 2018). Hence, BME induction of EZH2 in BMMs may trigger an epigenomic disturbance beyond H3K27me3.

The loss of ER expression during BoM appears to be transient. In the advanced stage when the osteolytic vicious cycle starts (Boyce et al., 1999; Kozlow and Guise, 2005; Weilbaecher et al., 2011), ER expression may recover, perhaps driven by the stimulatory effects of other stromal cells recruited to macro-metastases. The positive spatial correlation between RANK and ER supports this possibility. However, the BME-conferred endocrine resistance may persist in cells maintaining interactions with OGs suggesting that additional mechanisms may be involved (Eyre et al., 2019). Thus, overt BoMs may be heterogeneous, including a subset whose ER signaling remains repressed, which may be responsible for the rapid development of resistance observed during metastatic treatments (Johnston, 2010).

Although our experiments focused on BoMs, we are not ignoring the fact that other metastases also need to be prevented and cured. Recent genomic analyses revealed frequent metastasis-to-metastasis seeding (Brown et al., 2017; Ullah et al., 2018), suggesting that bone may not be the final destination for cancer cell dissemination. In fact, over two-thirds of bone-only metastases subsequently develop other metastases (Coleman, 2001). The enhancement of stem cell signaling in BME raises the possibility that bone may invigorate DTCs for further metastases, and this possibility has recently gained support in our co-submitted manuscript (Zhang et al., 2021). Therefore, investigations on BoMs may have much broader impacts.

Limitations of study

Our data did not address the question of whether the observed effects are specific to the BME. It is possible that in non-bone organs similar mechanisms also lead to increased phenotypic plasticity via other cell types. Our study is also limited by the lack of naturally occurring murine ER⁺ models that recapitulate endocrine responses and development of resistance, as well as inability to stably tag all PDX models for lineage tracing.

STAR★METHODS

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QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.devcel.2021.03.008>.

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AUTHOR CONTRIBUTIONS

Conceptualization, I.L.B., W.Z., M.W.G., S.H., H.W., P.S., and X.H.-F.Z.; methodology, I.L.B., W.Z., H.W., P. Sarkar, Z.G., Z.X., Z.G., Z.L., Y.-W.W., J.L.,

(H) PET-CT images showing 18F-FDG uptake in spontaneous BoMs (hind limbs) following single agent (fulvestrant) or combination (EPZ > fulvestrant) treatment. (n = 4 mice). Error bars: mean ± SD.

(A-H) p values were computed by two-tailed unpaired Student's t tests unless otherwise noted.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Estrogen Receptor alpha (D8H8)	Cell Signaling Technology	Cat# 8644S; RRID: AB_2617128
EZH2 (D2C9)	Cell Signaling Technology	Cat# 5246S; RRID: AB_10694683
Red Fluorescent Protein	Rockland-Fisher	Cat# 600-401-379; RRID: AB_2209751
Beta-actin (8H10D10)	Cell Signaling Technology	Cat# 3700S; RRID: AB_2242334
Cytokeratin 19 (BA-17)	Genetex	Cat# GTX27755; RRID: AB_369536
FGF2 (C-2)	Santa Cruz	Cat# sc-74412; RRID: AB_1122854
Human PDGF R beta Antibody	R&D Systems-Fisher	Cat# AF385-SP; RRID: AB_355339
Fgf Receptor 1 (D8E4) XP	Cell Signaling Technology	Cat# 9740S; RRID: AB_11178519
Human Vimentin (VIM-V9-L-CE)	Leica	Cat# VIM-V9-L-CE; RRID: AB_442141
Vimentin (D21H3)	Cell Signaling Technology	Cat# 5741S; RRID: AB_10695459
PDGF Receptor β (28E1) Rabbit mAb	Cell Signaling Technology	Cat# 3169S; RRID: AB_2162497
Estrogen Receptor alpha Monoclonal (6F11)	Thermo Fisher Scientific	Cat# MA1-80216; RRID: AB_930763
GFP (Htz-GFP19C8)	Sloan-kettering	RRID: AB_2716737
GFP (Htz- GFP19F7)	Sloan-kettering	RRID: AB_2716736
Cytokeratin 8 (k8)	DSHB	Cat# TROMA-I-s; RRID: AB_531826
Donkey Anti-Rat IgG 594	Jackson ImmunoResearch	Cat# 111-585-144; RRID: AB_2307325
Donkey Anti-Mouse IgG 647	Jackson ImmunoResearch	Cat# 715-605-151; RRID: AB_2340863
Goat Anti-Rabbit IgG 488	Jackson ImmunoResearch	Cat# 712-546-153; RRID: AB_2340686
Tri-methyl-Histone H3 (Lys27) Antibody	EMD Millipore	Cat# 07-449; RRID: AB_310624
Tri-methyl-Histone H3 (Lys27) Antibody	Cell Signaling Technology	Cat# C36B11; RRID: AB_11220433
Donkey Anti-Rabbit IgG 488	Jackson ImmunoResearch	Cat# 711-545-152; RRID: AB_2313584
PR Antibody (H-190)	Santa Cruz Biotechnology	Cat# Sc-7208; RRID: AB_2164331
Total Histone 3	Biolegend	Cat# 819412; RRID: AB_2820128
Acetyl-Histone H3 (Lys27) (D5E4) XP	Cell Signaling Technology	Cat# 8173S; RRID: AB_10949503
Alkaline Phosphatase	Abcam	Cat# ab108337; RRID: AB_10862036
Tri-Methyl-Histone H3 (Lys4) (C42D8)	Cell Signaling Technology	Cat# 9751S; RRID: AB_2616028
Tri-Methyl-Histone H4 (Lys20) (D84D2)	Cell Signaling Technology	Cat# 5737S; RRID: AB_10828431
Tri-Methyl-Histone H3 (Lys36) (D5A7) XP	Cell Signaling Technology	Cat# 4909S; RRID: AB_1950412
Tri-Methyl-Histone H3 (Lys9) (D4W1U)	Cell Signaling Technology	Cat# 13969S; RRID: AB_2798355
Bacterial and virus strains		
XL1-Blue	Agilent	Cat# 200236
Stbl3	Thermo Fisher Scientific	C737303
Biological samples		
PDX HCI011	Gift from Dr. Alana L. Welm's Laboratory, University of Utah	N/A
PDX WHIM9	Gift from Dr. Matthew Ellis's Laboratory, Baylor College of Medicine	N/A
Chemicals, peptides, and recombinant proteins		
Human FGF-2	Miltenyi Biotec	Cat# 130093838
Human PDGF-BB	PeproTech-Fisher	Cat# 100-14B
Human PDGF-CC	PeproTech-Fisher	Cat# 100-00CC
Human PDGF-DD	R&D Systems	Cat# 1159-SB-025
β -Estradiol	Sigma-Aldrich	Cat# E2758

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Streptavidin MyOne T1 Dynabeads		N/A
Biotinylated protein L		N/A
Cycloheximide, ultrapure, 98%	Alfa Aesar™-Fisher	Cat# J6690103
1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC)	1,2-dihexanoyl-sn-glycero-3-phosphocholine	Cat# 850305P-500 mg
Fulvestrant	Selleck Chemicals	Cat# S1191
4-Hydroxytamoxifen (4-OHT)	Sigma-Aldrich	Cat# H6278-10MG
EPZ-011989-9	Gift from Epizyme	N/A
Anti-Fibroblast MicroBeads, human	Miltenyi Biotec	Cat# 130-050-601
BGJ398 (NVP-BGJ398)	Selleck Chemicals	Cat# S2183
FK506 (Tacrolimus)	ApexBio Technology LLC	Cat# B2143
Carbenoxolone disodium (CBX)	Tocris	Cat# 3096
Sunitinib	Selleckchem-Fisher	S7781
D-Luciferase	Goldbio	Cat# LUCNA-1G

Critical commercial assays

Nextera XT DNA Library Prep Kit	Illumina	Cat# FC-131-1096
Nextseq 500/500 high output v2 Kit	Illumina	Cat# FC-404-2002
CellTrace™ Far Red Cell Proliferation Kit	Thermo Fisher Scientific	Cat# C34572
iScript™ Reverse Transcription Supermix	Bio-rad	Cat# 1708841
Lipofectamine RNAiMAX Transfection Reagent	Thermo Fisher Scientific	Cat#13778030
iBlot® Transfer Stack, nitrocellulose, mini	Thermo Fisher Scientific	Cat# IB301032
NuPAGE™ Novex™ 4-12% Bis-Tris Protein Gels, 1.5 mm, 15-wel	Thermo Fisher Scientific	Cat# NP0336BOX

Deposited data

SuperSeries for all sequencing datasets	This Paper	GSE160583
MCF7-MSC TRAP-sequencing dataset	This Paper	GSE137270
Human osteoblast dataset	This Paper	GSE137245
MCF7 bone metastasis evolving barcode dataset	This Paper	GSE160566
Bulk-ATAC-seq SCP2 dataset	This Paper	GSE160582
Whole exome sequencing (WES)	This Paper	GSE161181

Experimental models: cell lines

Human breast cancer MCF7	ATCC	Cat# HTB-22, RRID:CVCL_0031
Human breast cancer T47D	ATCC	Cat# HTB-133, RRID:CVCL_0553
Human breast cancer ZR75-1	Gift from Dr. Rachel Schiff's Lab, Baylor College of Medicine	Cat# CRL-1500, RRID:CVCL_0588
Human breast cancer ZR75-30	ATCC	Cat# CRL-1504, RRID:CVCL_1661
Human breast cancer MDA-MB-361	ATCC	Cat# HTB-27, RRID:CVCL_0620
Human pre-osteoblast FOB-1.19	ATCC	Cat# CRL-11372, RRID:CVCL_3708
Human mesenchymal stem cell MSC	Gift from Dr. Max Wicha's Laboratory, University of Michigan	N/A
Human Bone Marrow	Lonza	Cat# 2M-125C
Human pre-osteoclast U937	ATCC	Cat# CRL-1593, RRID:CVCL_0007
Mouse pre-osteoblast MC3T3-E1	ATCC	Cat# CRL-2953, RRID:CVCL_0409
Human bone marrow fibroblast Hs5	ATCC	Cat# CRL-11882, RRID:CVCL_3720
Human primary cells HCI011	This paper	N/A

Experimental models: organisms/strains

Athymic Nude mice [Athymic Nude-Foxn1 nu]	Envigo	N/A
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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Scid/Beige mice [C.B-17/lcrHsd-Prkdc scid Lyst bg-J]	Envigo	N/A
Osx1-GFP-cre/IDTR	This paper	N/A
Oligonucleotides		
Silencer™ Negative Control	Thermo Fisher Scientific	AM4611
EZH2 siRNA (human) - ID: s4918	Thermo Fisher Scientific	4392420
EZH2 siRNA (human)- ID: s4916	Thermo Fisher Scientific	4392420
Oligos for qPCR	This paper (Table S1)	N/A
Recombinant DNA		
3X ERE TATA luciferase	A gift from Dr. Donald McDonnell to Addgene, Duke University	Plasmid #11354, RRID:Addgene_11354
GFP-RPL10	Dr. William T. Pu' Laboratory, Harvard Stem Cell Institute	NA
pMD2.G	A gift from Didier Trono to Addgene, EPFL	Plasmid# 12259, RRID:Addgene_12259
psPAX2	A gift from Didier Trono to Addgene, EPFL	plasmid #12260, RRID:Addgene_12260
Pwpt-Fluc/GFP	Wang et al., 2015	N/A
Pwpt-Fluc/RFP	Wang et al., 2015	N/A
ERE-pWPXL/GFP	This paper (modified from Plasmid # 12257; gift from Didier Trono to addgene) EPFL	N/A
hgRNA-A21_pLKO-Puro	A gift from George Church, Harvard (Addgene plasmid # 10057)	Plasmid # 100570. RRID:Addgene 100570
Lenti-iCas9-neo	A gift from Qin Yan, Yale (Addgene plasmid # 85400)	Plasmid # 85400. RRID:Addgene_85400
Software and algorithms		
Expanding Ploidy and Allele-Frequency on Nested Subpopulations	Stanford University	https://CRAN.R-project.org/package=expands
Evolving barcode analysis	This Paper	https://github.com/LiuzLab/ER_positive_breast_cancer-manuscript
TraceQC	Baylor College of Medicine	https://github.com/LiuzLab/TraceQC/
Rstudio	Rstudio	http://www.rstudio.com/
Galaxy project	Penn State, Johns Hopkins University, Oregon Health & Science University, and the Galaxy Community	http://galaxyproject.org/
ImageJ	National Institute of Health	https://fiji.sc/
Flow Jo V10.0	FlowJo, LLC	https://www.flowjo.com
LAS AF Lite	Leica Microsystems	RRID:SCR_013673
Living Image 4.5.2	PerkinElmer	https://www.perkinelmer.com/lab-products-and-services/resources/in-vivo-imaging-software-downloads.html
Inveon Research Workplace (IRW)	SIEMENS	https://www.siemens-healthineers.com/en-us/molecular-imaging/preclinical-imaging/inveon-workplace/
Graphpad Prism	GraphPad Software, Inc.	https://www.graphpad.com/scientific-software/prism/
Combeneft	Cancer Research UK Cambridge Institute	http://sourceforge.net/projects/combeneft/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact Dr. Xiang H.-F. Zhang at xiangz@bcm.edu

Material availability

Plasmids and cell lines generated in the study will be made available upon request.

Data and code availability

Datasets were deposited in Gene Expression Omnibus (Edgar et al., 2002), with the following GEO accession numbers (GSE137245; GSE137270, GSE160566, GSE160582, and GSE161181). The GEO Reference Series connecting all datasets is GSE160583. Barcode analysis pipeline is accessible at: https://github.com/LiuzLab/ER_positive_breast_cancer-manuscript.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animal studies

All animal experiments were in compliance with Institutional Animal Care and Use Committee of Baylor College of Medicine. Nude mice [Athymic Nude-Foxn1^{nu}] and SCID/Beige mice [C.B-17/lcrHsd-Prkdc scid Lyst bg-J] were purchased from Envigo. Osx1-GFP-cre/iDTR was generated from Osx1-GFP-cre [B6.Cg-Tg(Sp7-tTA,tetO-EGFP/cre)1Amc/J] and STP-iDTR mice [C57BL/6-Gt(ROSA)26Sortm1(HBEGF)Awai/J] originally obtained from Jackson Laboratory. For all in vivo experiment, 4- to 7-week-old female mice were used.

Patient-derived xenografts (PDXs) and primary cells

ER+ PDX models were kindly provided by Alana L. Welm (HCI011) and Matthew Ellis (WHIM9). All PDXs were maintained in SCID/Beige mice. PDX-HCI011 primary cell line was successfully generated from freshly harvested orthotopic tumors in RPMI medium supplemented with 15–20% FBS, 1X antibiotics Penicillin/Streptomycin and 1X antimycotic Amphotericin (Gibco#15240062). Media was changed every 48 h.

Cell lines

Human estrogen receptor positive (ER+) breast cancer cell lines MCF7, T47D, MDA-MB-361 and ZR75-30, pre-osteoblast cells hFOB-1.19, mesenchymal stem cells MSC, pre-osteoclast U937, and the mouse pre-osteoblast MC3T3-E1 were purchased from American Type Culture Collection (ATCC). The Human ER+ breast cancer cell line ZR75-1 was kindly provided by Dr. Rachel Schiff. MCF7 single cell derived populations M7-SCP1, M7-SCP2, M7-SCP3, and M7-SCP4 were generated from single clones of parental MCF7 cells. The mouse cell line AT3 was a kind gift of S.I. Abrams at Roswell Park Cancer Institute.

METHOD DETAILS

Ovariectomy and aromatase inhibition

Mouse ovaries were removed using the previously described surgical procedure (Ström et al., 2012). Briefly, mice were anesthetized with 2% isoflurane, and placed on a temperature-regulated heat pad. The dorsal area covering the lumbar vertebrae was shaved to display a 3x3 cm patch and disinfected. A 1 cm mid incision was performed on the skin and a 0.5 cm incision in the peritoneum allowed access to the ovary. Each ovary was cauterized and removed and each peritoneum was closed using an absorbable suture (Ethicon Vicryl #J497G). Skin closure was completed using EZ clips (Stoelting # 59027) and mice were monitored for recovery. Paracrine estrogen was blocked with oral administration of 0.5 mg/kg Letrozole as previously described (Brodie et al., 2005).

Intra-iliac-artery (IIA) and mammary fat pad (MFP) injections

Intra-iliac-artery (IIA) injection was performed as previously described (Yu et al., 2016). Briefly, breast cancer cells were trypsinized, pelleted, washed twice with PBS, collected in cold PBS and kept on ice. For established breast cancer cell lines such as MCF7 and ZR75, 5x10⁵ cells were injected into the internal iliac artery to generate bone lesions. For PDX models, 2x10⁵ cells were injected except when specified otherwise. For the syngeneic and aggressive model AT3, 1x10⁵ cells were injected. *Mammary fat pad (MFP)* injections were performed as previously described (Zhang et al., 2019a). Cells were prepared as for IIA injection. For all xenograft models except spontaneous metastasis from PDX-HCI011 (Figure 7), estrogen was provided through drinking water to reduce deleterious side effects. Based on previous studies, 8 µg/ml of 17-β-estradiol was added to mouse water bottles and replaced twice a week (Levin-Allerhand et al., 2003; Welsch et al., 1981). In experiments involving dual IIA and MFP injections as in Figure 1D, the same cell number was injected to bone and mammary gland, respectively.

Spontaneous metastasis from PDX models

To evaluate spontaneous metastases from PDX-HCI011, Foxn1^{nu} mice were orthotopically transplanted with 5×10^5 cells from freshly dissociated and purified tumor (Mouse Cell Depletion Kit; miltenyibiotec #130-104-694). Estrogen pellets were subcutaneously implanted to increase the tumor take rate. When tumors reached 1x1 cm, a survival surgery was performed to remove both the primary tumors and the remaining estrogen pellets. A three-week treatment with vehicle or EPZ011989 (125 mg/kg; oral gavage; twice daily) was started two weeks after orthotopic tumor removal. Then, 250 mg/kg of fulvestrant was administered weekly via subcutaneous injection to both EPZ011989 and vehicle pretreated groups for 3 consecutive weeks. Mice were monitored for 3 months before 18F-Fluorodeoxyglucose positron emission tomography (PET) and computed tomography (CT) scans were performed.

Drug treatments

In vivo

The selective estrogen receptor degrader (SERD) fulvestrant (Selleckchem #S1191) was solubilized in 5% DMSO and 95% corn oil and administered subcutaneously at 250 mg/kg per mouse, once a week for 2 consecutive weeks. Letrozole was purchased from Selleckchem (#S1235), diluted in 0.5% sodium carboxymethyl cellulose (NaCMC), and administered at 0.5 mg/kg via oral gavage. EPZ-110989 was kindly provided by Epizyme and stock solutions were prepared following the company's recommendations, using 0.5% sodium carboxymethyl cellulose (NaCMC) and 0.1% Tween-80 as vehicle. A dosage of 125 mg/kg of EPZ-011989 or vehicle was administered twice daily by oral gavage for 3 weeks.

In vitro

Tamoxifen and fulvestrant were used *in vitro* at a concentration of 100 nM and 20 nM per well, respectively. 1–4 μ M EPZ011989 was used for coculture experiments. For calcium signaling studies, we used 1 μ M of GAP19 (cat#5353) to inhibit gap junction (CX43), and 1 μ M of FK506/Tacrolimus (cat#S5003) to block calcineurin. Similarly, 2.5 μ M BGJ398 (selleckchem #S2183) and 10 μ M Sunitinib (Selleckchem #S7781) were used to inhibit FGF receptors and PDGF receptor B, respectively.

Immunohistochemistry, immunofluorescence, and immunoblotting

IHC/IF

Tissues were processed with the help of the Breast Center Pathology Core at Baylor College of Medicine. Immunohistochemistry staining and immunoblotting were performed using antibodies against human ER α (D8H8 and 6F11), Progesterone Receptor (H-190), EZH2 (D2C9), Cytokeratin 8 (TROMA-I), α -Smooth Muscle Actin (D4K9N), Vimentin (D21H3), RANK (64C1385), ALP (ab75635), FGF2 (C2, #sc-74412), CD31 (AF3628), PDGFR β (28E1), H3K27me3 (C36B11). Western blot: Protein extraction was performed using RIPA buffer as previously described (Rajapaksa et al., 2015). Protein electrophoresis and transfer were performed using the XCell SureLock and the iblot system (Invitrogen), respectively. Immunoblotting was performed using antibodies against Estrogen Receptor α (D8H8), Cytokeratin 19 (BA-17), Red Fluorescent Protein (Rockland-Fisher), β -actin (8H10D10), H3K27me3 (Millipore Cat# 07-449), H3K36me3 (D5A7), H3K9me3 (D4W1U), H4K20me3 (D84D2), H3K4me3 (C42D8), and Histone 3 (D1H2). Images were captured using the Odyssey system (Li-cor).

Image acquisition and quantification

Images were acquired with the Leica TCS SP5 or the Zeiss LSM 880 with Airyscan FAST Confocal Microscope. A 40x oil objective lens (Immersion oil refractive index $n=1.51$) was used to capture images for immunofluorescence quantifications except when specified otherwise. We compared only sets of images that were captured under the same microscopic setting. To further reduce technical biases and batch effects, compared samples were processed and stained in parallel. All images were quantified using ImageJ 1.52i. All statistical analyses represent a two-tailed unpaired Student's *t* test except when specified otherwise. Whenever applicable (Figures 1E, 1F, 3A–3C, 3E, 3G, 6E, 6K, 6L, 6P, and 7D), single cells from at least 3 biological replicates were used for statistical analysis. Figures 2J, and 6D are representative of at least 3 independent lesions.

Tumor classification

Based on size

metastases were classified based on cell number. On average, small lesions were defined as lesions with fewer than 100 cells. The average maximum cell number common among all models was ~ 150 for macrometastases (large lesions) and ~ 90 for micrometastases (small lesions) which gives a fold change superior to >1.5 between the two experimental stages of metastasis. The tumor size in different models (HCI011, WHIM9, MCF7, ZR75-1 and M7-SCP2) of bone metastasis was variable due to differences in tumor aggressiveness. Accordingly, we used a cutoff of median ± 0.5 S.D. as a more consistent variable to segregate tumors into micrometastasis and macrometastasis. *Based on location*: cancer cells were classified as proximal if they were directly interacting with the bone matrix or separated from it by less than two cells (≤ 2 cell distance), or classified as distal if separated from the bone matrix by 3 cells or more (≥ 3 cell distance).

Recombinant protein and calcium treatments

All experiments involving protein recombinants were performed in low serum media (2% serum). Protein recombinants for FGF2 (#130093838), PDGF-BB (100-14B), PDGF-CC (100-00CC), PDGF-DD (1159-SB-025) were diluted in PBS and used at a concentration of 20 or 100 ng/ml. To evaluate endocrine resistance after treatment with FGF2 and PDGF recombinants, cells were starved for

48 h in 2% charcoal stripped media before a 20 nM fulvestrant treatment. All experiments involving cells growth were performed in 3D culture and bioluminescence was assessed 72 h post treatment. For western blot, short term treatments were performed for 24 h and long-term ones for up to 72 h. Western blot experiments were performed in 2D in most cases, except when specified otherwise. *Calcium treatment*: 2×10^6 cells (MCF7 or ZR75-1) were cultured in regular medium for 24 hours. Regular medium was replaced with calcium-free minimum essential medium (S-MEM) and treated with vehicle or 2 mM Calcium chloride for 24 h. After collecting all cells, we extracted protein lysates to assess the effect of calcium on ER expression by western blot.

Live imaging

For in vivo experiments, all cells were pre-labelled with Luciferase fused to GFP or RFP as previously described (Wang et al., 2015). 5×10^5 breast cancer cells were injected in bone or mammary fat pad, except when specified otherwise. Tumor growth was monitored using the IVIS Lumina II system. Briefly, mice were anesthetized in an isoflurane chamber (2%) and 100 μ l of D-Luciferin was administered through retro-orbital injection to each mouse before image acquisition. For in vitro experiments, 10,000 cells were plated in low attachment 96 well plates to assess cell growth at 72 or 96 h post-treatment. For conditions demanding estrogen depleted media and starvation, 20,000 cells were cultured per well. Images were acquired after adding 1X concentration of D-luciferin containing media to each well.

Reverse phase protein arrays (RPPA)

MCF7 and M7-SCP2 cell lines were injected to bone using intra-iliac artery injection. After 5 weeks of metastasis formation, bones were collected in aseptic conditions and dissociated to generate bone-entrained MCF7-Bo and M7-SCP2-Bo cell lines. Cells were cultured in DMEM 10% FBS supplemented with 1X antibiotics (penicillin, streptomycin) and antimycotics (amphotericin). Bone-educated cells were FACS-sorted and maintained in 2D culture. Approximately 5×10^6 non-entrained and bone-entrained MCF7 and M7-SCP2 cells were harvested in freshly prepared RPPA lysis buffer containing protease and phosphatase inhibitors. Protein lysate was cleared twice via centrifugation (14,000 g for 15 min at 4°C). A BCA assay was adopted for protein quantification (Thermo-Fisher #23225). All samples were diluted in RPPA solution and SDS to a final concentration of 0.5 mg/ml and heated for 8 min at 100°C for protein denaturation. RPPA was performed as previously described (Welte et al., 2016). In brief, samples and control lysates were spotted onto nitrocellulose-coated slides (Grace Bio-labs; array format of 960 lysates/slide or 2880 spots/slide). The automated slide stainer Autolink 48 (Dako) was used to probe 236 antibodies (against total and phospho-proteins) on slides. Control slides were incubated with antibody diluent. A biotinylated secondary antibody was probed by streptavidin-conjugated IRDye680 fluorophore (LI-COR Biosciences) and total protein was detected with Sypro Ruby Protein Blot Stain according to the manufacturer's instructions (Molecular Probes). All slides were scanned on a GenePix 4400 AL scanner and images were analyzed with GenePix Pro 7.0 (Molecular Devices). Samples were normalized as previously described (Chang et al., 2015). After quality control 233 antibodies remained and were used for subsequent data analysis.

PET/CT imaging and analysis

Radiopharmaceuticals and small-animal PET-CT

Fluorine-18 labeled fluorodeoxyglucose (18F-FDG), fluoroestradiol (18F-FES), and sodium fluoride (18F-NaF) was purchased from (Cyclotope, Houston, TX). All CT and PET images were acquired using an Inveon scanner (Siemens AG, Knoxville, TN). The mice were injected with 9.25 MBq (250 μ Ci) of FES and 11.1 MBq (300 μ Ci) of either 18F-FDG or 18F-NaF radiotracers at any given time. To identify skeletal metastases or measure tumor metabolic activities, 18F-NaF or 18F-FDG were injected intraperitoneally, and to measure estrogen activity 18F-FES was injected intravenously via tail vein. Before 18F-FDG administration, the mice were fasted for approximately 12 h. PET and CT were performed 1 h after injection of radioisotopes. During imaging, a respiratory pad was placed under the abdomen of the animal to monitor respiration (Biovet, Newark, NJ). Mice were anesthetized with isoflurane gas (1–3%) mixed with oxygen at a flow rate of 0.5–1 L/minute, and adjusted accordingly during imaging to maintain normal breathing rates. A CT scan was acquired with the following specifications: 220 acquired projections except for the 18F-NaF imaging which was 360 full scan. Each projection was 290 ms with x-ray tube voltage and current set at 60 kVp and 500 μ A, respectively. A 30-minute PET scan was immediately acquired afterward. The PET scans were reconstructed using OSEM3D reconstruction method and registered to the CT scan for attenuation correction. *PET Image Analysis*: The PET images were quantified using Inveon Research Workspace IRW (IRW, Siemens AG, Knoxville, TN). Using the reconstructed PET scan, bone (hind limbs) and mammary fat pads were manually selected to form regions of interest (ROI) on the PET-CT images. The data were represented as standardized uptake value (SUV) normalized to body weight. For PDX spontaneous metastasis to bone, a 90% SUVmax thresholding was applied to ROI.

microCT imaging and analysis

A microPET/CT scanner (Siemens Medical Solutions USA, Inc; Malvern, PA; USA) was used to acquire microtomography images. Paired murine hindlimb specimens with bone metastases were imaged with a spatial resolution of 20 microns. Images were converted to Dicom format using Inveon software (version 4.2; Siemens) and bone analyses (volume and mineral density) and three-dimensional reconstruction/visualization performed using Skyscan CTAn and CTvox software packages by Bruker (version 1.19 and 2.3.2; Kontich; Belgium).

Mammosphere and coculture assays

5x10⁵ cells were plated in low attachment 6 well plates (Greiner) using regular (10% FBS) of serum free DMEM/F12 media supplemented with 2–3% dextran-coated Charcoal stripped. We used a 1/1 ratio for cocultures between cancer cells and stromal cells except when specified otherwise. Cells were collected after 24, 48, or 72 h of culture for downstream analyses. For immunofluorescence, cells were fixed with 2% PFA for 24 h, washed 3 times with PBS, embedded in paraffin, and sectioned for imaging.

Quantitative real-time PCR

Total RNA was extracted using the Direct-zol Zymo according to the manufacturer protocol. Copy DNA was synthesized using the iScript cDNA Synthesis Kit (Biorad). All primers are indicated in Table S1. Real-time PCR was performed on the CFX connect system (Biorad) using PowerUP SYBR Green master mix (Thermo Fisher, #A25780) for amplification.

Bulk ATAC sequencing (ATAC-seq) and analysis

ATAC-seq assay

ATAC-seq was performed as previously described (Buenrostro et al., 2015). Here, we collected 50,000 cells from parental (M7-SCP2) and bone-entrained (M7-SCP2-Bo) cells at different passages (#2, #4, #6 and #12). DNA transposition was performed on freshly collected cells using the Nextera Tn5 Transposase from Illumina. Purified DNA was stored at -80 °C for each passage before library preparation. All experiments were performed in parallel in both parental and bone-entrained cells.

ATAC-seq analysis

Peak generation pipeline

Analysis was conducted with a modified version of the Encode Consortium's ATAC-Seq Pipeline. Adapters were trimmed from input FASTQ files using cutadapt. Alignment was performed using Bowtie2. Samtools and Picard were used for post alignment filtering to remove duplicate, unmapped, and mitochondrial reads. Pseudo-replicates were generated for both individual replicates and pooled replicates by randomly dividing the input into two equal length files. The MACS2 peak caller was utilized to generate peaksets for all true replicates and pseudo-replicates. Peaks with p value < 10⁻⁵ were retained for further analysis. Peaks in the Encode DCC consensus blacklist regions were also removed. Then, pairwise comparisons between each pair of biological replicates, the two pseudo-replicates generated from the pooled replicate file, and pseudo-replicates generated from each replicate were conducted. The Encode IDR (irreproducible discovery rate) was used to rank the consistency of each peak region; only peaks with IDR<0.05 were retained. Further analysis was conducted using the overlap IDR thresholded peaks between pooled pseudo-replicates.

Post-peak analysis

A consensus peakset was then generated by merging all the peak regions for the samples of interest using BedTools (Quinlan and Hall, 2010). Promoter regions were retrieved from the UCSC genome browser and were defined as 5000 bases up and 1000 bases down from the TSS. Heatmaps and profile plots for the peak and promoter regions were generated using the DeepTools (Ramírez et al., 2014) utility. The IGV (Thorvaldsdóttir et al., 2013) utility was utilized to generate visualizations for specific gene regions. Further analysis utilized the DiffBind (Stark and Brown, 2011) suite to identify differentially accessible regions (DAR). Both promoter and consensus peak regions were used as peaksets for occupancy and differential binding affinity analysis. DiffBind utilizes DeSeq2 to identify and calculate log fold change and p values for DAR. Contrasts were established between the MCF7 parental and MCF7-Bo samples and the M7-SCP2 and M7-SCP2-Bo samples while controlling for passage number as a confounding factor. DiffBind was also used to generate the PCA and Volcano Plots. DAR generated from Diffbind were then labelled with genes based on the nearest TSS using HOMER (Heinz et al., 2010). HOMER's findMotifGenome module was also utilized to conduct motif analysis. The input file consisted of regions with lower binding in M7-SCP2-Bo compared to M7-SCP2 with an FDR cutoff of <0.05. A region size of 200 and the masked genome setting were used. All other settings used the default HOMER options.

Whole-exome sequencing (WES)

WES library was prepared using the Nextera DNA exome kit (Illumina # 20020616) per manufacturer's instructions and sequenced on a Novaseq 6000 platform at ~100x depth (paired end 100bp, 50 million reads per sample). FastQC and mulitQc were used for quality control. After adaptor removal using cutadapt and trim galore, reads were aligned to reference genome (hg19) using BWA-MEM. BAM files were filtered, and duplicate reads removed using samtools (Li et al., 2009) and Picard. A normal whole exome sequencing sample was downloaded for 1000-Genomes (ERR031938), aligned to the reference genome (hg19), and downsampled (<http://broadinstitute.github.io/picard/>). We generated pileups from BAM files using samtools mpileup. Varscan 2 was used to call copy numbers and somatic mutations (Koboldt et al., 2012, 2013). Only variant calls with p value < 10⁻² were used for downstream analyses. Data processing was performed on public server (Afgan et al., 2018). To evaluate the heterogeneity and subclonality of all M7-SCP cells, we used the Expands package (<http://cran.r-project.org/web/packages/expands/>). For each sample, a Z-score analysis was performed using the matrix of predicted subpopulation. Dominant subpopulations with a positive Z-score were used to assess tumor heterogeneity represented by pie charts. Tumor purity was estimated based on cellular frequencies of the largest subpopulation as previously described (Andor et al., 2014).

Histone protein post-translational modification (PTM) analysis

Un-entrained (MCF7 and M7-SCP2) cells were either directly purified from bone (Passage 0: P0) or purified from bone and cultured in vitro for 10 passages (P10). Each sample was washed 3 times with PBS and snap frozen pellets were shipped to Active Motif for PTM quantitation (www.activemotif.com). Briefly, a pilot study was performed to determine the optimal histone extraction method for the samples. Using the additional 6 samples, three lysis methods were evaluated: 1) A one-step method wherein histones are acid extracted directly from the frozen cell pellet, 2) A two-step method wherein a sucrose-based hypotonic buffer is used to lyse the cells and histones are acid extracted from isolated nuclei, or 3) A two-step method wherein an IGEPAL-containing hypotonic buffer is used to lyse the cells and histones are acid extracted from isolated nuclei. Acid extraction was performed for 2 h at 4 °C, cellular debris was pelleted, and lysate aliquots were frozen in a methanol-dry ice bath and stored at -80 °C until testing. Histone yields for the three methods were evaluated using the Histone H3 Total bead and a two-fold five-point dilution series of the samples. The IGEPAL-containing hypotonic lysis buffer method gave the highest yield and was selected for use with the experimental samples. *Lysate Preparation:* Histones were extracted from the experimental samples using the method described above. Cellular debris was pelleted, and lysate aliquots were frozen in a methanol-dry ice bath and stored at -80 °C until testing. Next, relative histone H3 concentrations in the samples were determined using the H3 Total bead. Multiplex assays were performed with the beads of interest using sample volumes normalized for histone H3 concentration. *Assay protocol:* (1) Beads were added to wells in 25 μ l Assay Buffer supplemented with Inhibitor Cocktails (ABIC) for proteases, phosphatases and HDACs. (2) Samples as a four-point 1.4 dilution series were added to wells in 25 μ l ABIC in duplicate and incubated for 1 h at room temperature. (3) Three 100 μ l washes with 1X Wash Buffer (PBS containing 0.05% Tween-20) were performed using plate magnet to retain beads. (4) 50 μ l biotinylated Histone H3 antibody diluted 1:500 in Assay Buffer was added for the high abundance PTM multiplex assay for 1 h with agitation. 50 μ l biotinylated Histone H3 antibody diluted 1:250 in Assay Buffer was added for the low abundance PTM multiplex assay for 1 h with agitation. (5) Washes were performed as above. (6) 50 μ l of SAPE diluted 1:100 in Assay Buffer was added to each well and incubated for 30 min with agitation. (7) Beads were collected on a plate magnet and the SAPE solution discarded. (8) The assay plate was removed from the plate magnet and beads resuspended in 100 μ l 1X Wash Buffer and read on the Luminex LX100 Instrument. *Data analysis:* Histone H3 Total matched data sets were used to determine PTM/H3 ratios, the PTM percent change relative to each other and Student's t test p values.

Translating ribosome affinity purification (TRAP) sequencing (TRAP-seq)

TRAP assay was adopted from previous studies (Heiman et al., 2014). Here, we performed all experiments in 3D. We stably labeled MCF7 cells with GFP-RPL10a plasmids kindly provided by Dr. William Pu from Harvard. Cells were sorted using FACSaria II to enrich for GFP-positive cells. GFP-RPL10a-expressing MCF7 cells were maintained in 2% charcoal stripped medium for 48 h from which 1 million cells were seeded in 100 mm low attachment plates (Corning, cat #05-539-101) either alone or in coculture with human mesenchymal stem cells (MSCs). These cultures were incubated overnight and treated with 10 nM 17 β -estradiol, fulvestrant or 100 nM Tamoxifen for 24 h. Cells were collected for TRAP sequencing. Library was prepared using illumina Nextera XT Kit and paired-end sequencing was performed on a Nextseq 550 System. All sequencing experiments were performed at the Genomic and RNA Profiling core (GARP) at Baylor College of Medicine.

Tracing metastasis expansion in bone using CRISPR-Cas9/hgRNA system

CRISPR-Cas9 barcoding

The hgRNA A21 vector was previously characterized and published (Kalhor et al., 2017). MCF7 cells were stably infected with Lenti-iCas9-neo (Addgene #85400) and hgRNA-A21 (Addgene #100570) using Neomycin/puromycin antibiotic selection before intra-iliac artery injection (IIA). To activate cas9 expression, mice were administered 2 mg/kg of doxycycline via intraperitoneal injection at day 1 post-IIA. Doxycycline treatment was repeated once a week for 3 more weeks (see experimental design Figure 1A).

Laser capture micro-dissection (LCM) and barcode sequencing

Tumor bearing limbs (femur and tibia) were isolated, embedded in Tissue-Tek O.C.T., snap-frozen in liquid nitrogen, and stored at -80 °C until sectioning. 10 μ m cryosections of each bone were generated using Leica CM3050S cryotome equipped with a low-profile microtome blade. The chamber temperature was set at -26 °C. Sectioning was facilitated with the CryoJane Tape Transfer System and then placed on the PET membrane slides (MMI, Prod. No. 50103). Sections were fixed in ethanol and stained with DAPI and Arc-turusTM HistoGeneTM solution (Applied Biosystems) according to the instruction manual. Microdissection was performed using the Leica LMD7000 instrument. DNA was purified from each LCM-derived lesion using the Quick-DNA/RNA Microprep Plus Kit from Zymo (D7005). The evolving barcode library was generated as previously described (Kalhor et al., 2017). A paired-end sequencing was performed using the Hiseq 4000 system.

Bioinformatic analysis of the evolving barcode system

The R1 sequences were aligned and annotated using the TraceQC package (<https://github.com/LiuzLab/TraceQC>). First, the CRISPR barcode sequences were aligned to the hgRNA_A21 reference construct using the following score system: match +2, mismatch -2, gap opening -6, gap extension -0.1. After annotating the aligned sequences, the adapters were trimmed off and sequences with low alignment scores (<200) were filtered out. Sequences with less than 10 count were subsequently filtered out. TraceQC extracted mutation events from the sequence into 4 attributes: 1) the mutation type (insertion, deletion or point mutation), 2) the starting position of mutation, 3) the length of mutation, and 4) the altered sequence. We combined the mutation events for all the samples into a mutation count matrix and normalized samples using the read count per million (RPM) approach. The hierarchical

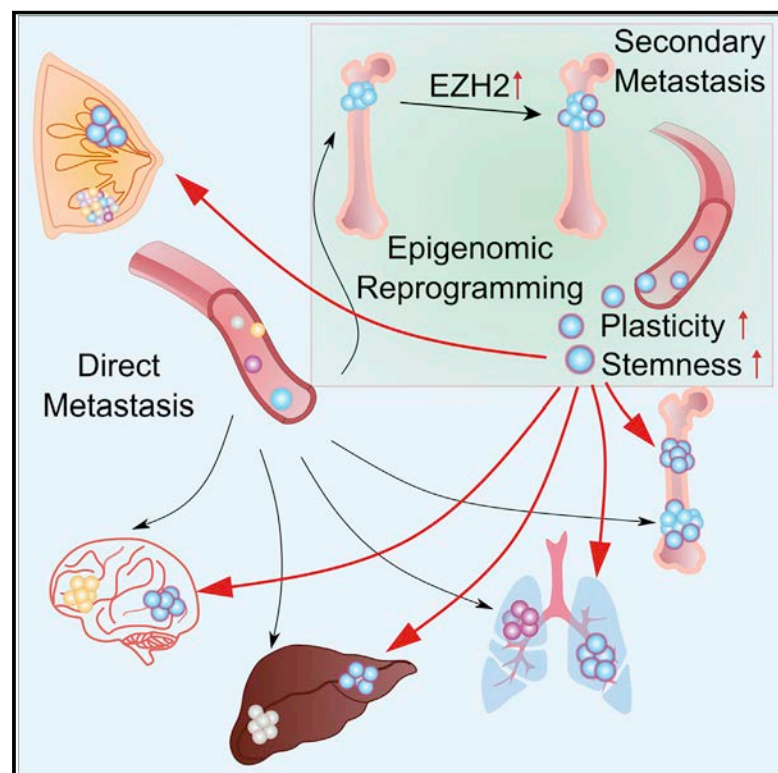
clustering based on mutations revealed 3 major modules. Within each module, the mutation count matrix was binarized into whether each mutation event exists in the samples or not: TRUE (mutation present) or FALSE (mutation absent). Then, we used maximum parsimony to establish the lineage relations within each module. To build the cell lineage network, we performed graphic LASSO using the Huge package. First, the mutation count matrix was Gaussianized using non-paranormal transformation provided by Huge package. Then, the graphic LASSO was applied to the Gaussianized mutation count matrix. We selected $\lambda = 0.52$ to make the graph have the maximum sparsity while remaining fully connected. Next, we applied a random walk-based community detection algorithm to detect the 3 modules in the graph. The algorithm is provided by the iGraph package. Detailed analysis pipeline is accessible at: https://github.com/LiuzLab/ER_positive_breast_cancer-manuscript.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data analysis was conducted in GraphPad Prism (v8.0.1) and R (version 3.4 R). Specific statistical approaches are indicated for each figure in the figure legend. For PET imaging, Mann Whitney *U*-test was used for statistical analysis. Pearson correlation was used for correlation studies.

The bone microenvironment invigorates metastatic seeds for further dissemination

Graphical abstract



Authors

Weijie Zhang, Igor L. Bado, Jingyuan Hu, ..., Chonghui Cheng, Zhandong Liu, Xiang H.-F. Zhang

Correspondence

xiangz@bcm.edu

In brief

Delineation of cancer cell spreading from primary bone metastasis by an evolving barcode system highlights the impact of the bone microenvironment in shaping secondary metastasis through mechanisms distinct from clonal selection.

Highlights

- Experimental bone metastases (BoM) spread to other organs in mouse models
- Evolving barcodes delineate further spread from BoM in spontaneous metastasis
- BoM spurs metastatic seeds with increased stemness and reduced organotropism
- EZH2 mediates secondary metastasis invigorated by the bone microenvironment

Article

The bone microenvironment invigorates metastatic seeds for further dissemination

Weijie Zhang,^{1,2,3,11} Igor L. Bado,^{1,2,3,11} Jingyuan Hu,^{4,5} Ying-Wooi Wan,^{4,6} Ling Wu,^{1,2,3} Hai Wang,^{1,2,3} Yang Gao,^{1,2,3} Hyun-Hwan Jeong,^{4,6} Zhan Xu,^{1,2,3} Xiaoxin Hao,^{1,2,3} Bree M. Lege,^{1,6} Rami Al-Ouran,^{4,7} Lucian Li,^{4,7} Jiasong Li,⁸ Liqun Yu,^{1,2,3} Swarnima Singh,^{1,2,3} Hin Ching Lo,^{1,2,3} Muchun Niu,⁹ Jun Liu,^{1,2,3} Weiyu Jiang,^{1,2,3} Yi Li,^{1,2,3} Stephen T.C. Wong,^{2,3,8} Chonghui Cheng,^{1,6} Zhandong Liu,^{4,6} and Xiang H.-F. Zhang^{1,2,3,10,12,*}

¹Lester and Sue Smith Breast Center, Baylor College of Medicine, Houston, TX 77030, USA

²Dan L. Duncan Cancer Center, Baylor College of Medicine, Houston, TX 77030, USA

³Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX 77030, USA

⁴Jan and Dan Duncan Neurological Research Institute, Texas Children's Hospital, Houston, TX 77030, USA

⁵Quantitative and Computational Biosciences Program, Baylor College of Medicine, Houston, TX 77030, USA

⁶Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA

⁷Department of Pediatrics, Baylor College of Medicine, Houston, TX 77030, USA

⁸Department of Systems Medicine and Bioengineering and Translational Biophotonics Laboratory, Houston Methodist Cancer Center, Houston, TX 77030, USA

⁹Integrative Molecular and Biomedical Sciences Graduate Program, Baylor College of Medicine, Houston, TX 77030, USA

¹⁰McNair Medical Institute, Baylor College of Medicine, Houston, TX 77030, USA

¹¹These authors contributed equally

¹²Lead contact

*Correspondence: xiangz@bcm.edu

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SUMMARY

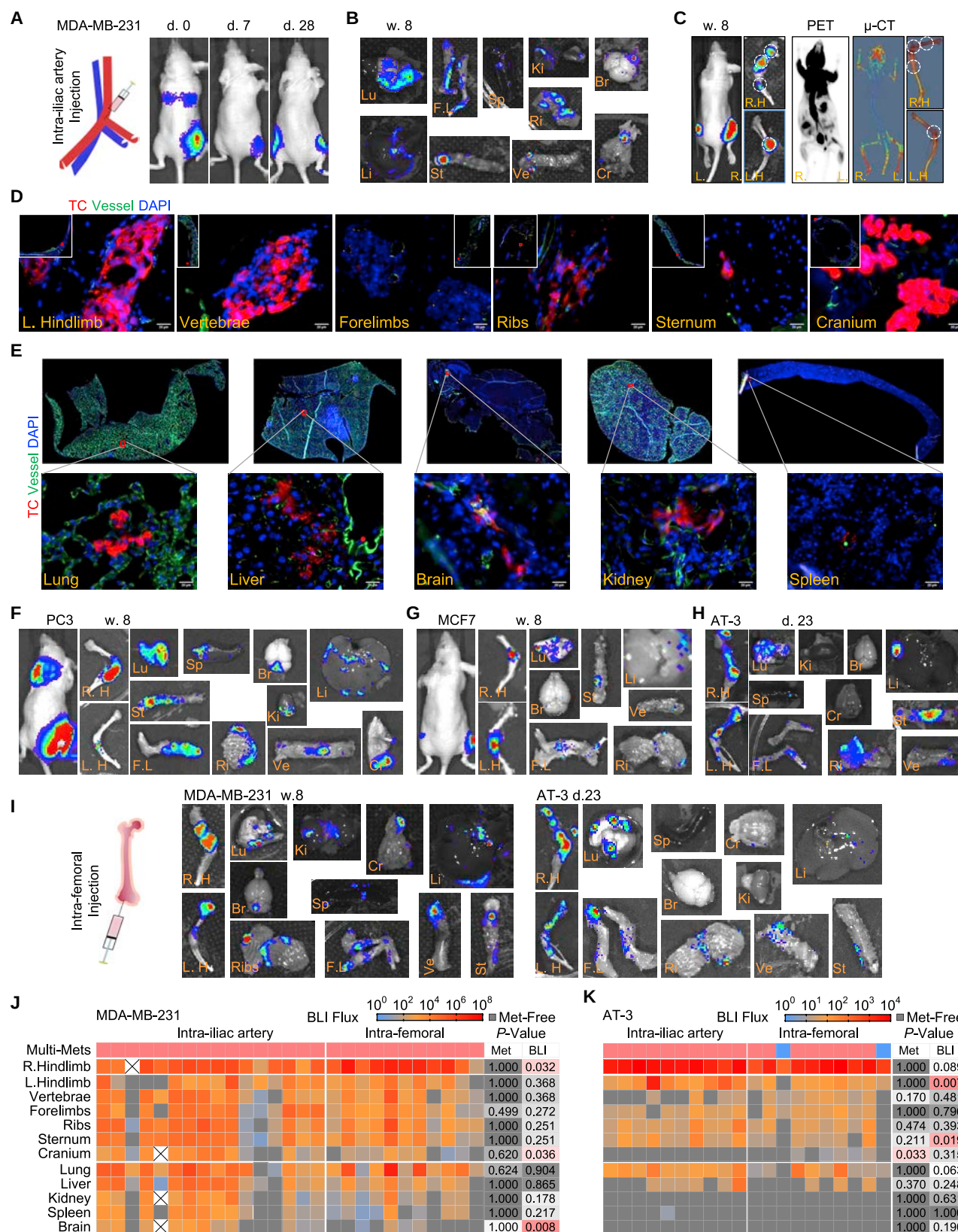
Metastasis has been considered as the terminal step of tumor progression. However, recent genomic studies suggest that many metastases are initiated by further spread of other metastases. Nevertheless, the corresponding pre-clinical models are lacking, and underlying mechanisms are elusive. Using several approaches, including parabiosis and an evolving barcode system, we demonstrated that the bone microenvironment facilitates breast and prostate cancer cells to further metastasize and establish multi-organ secondary metastases. We uncovered that this metastasis-promoting effect is driven by epigenetic reprogramming that confers stem cell-like properties on cancer cells disseminated from bone lesions. Furthermore, we discovered that enhanced EZH2 activity mediates the increased stemness and metastasis capacity. The same findings also apply to single cell-derived populations, indicating mechanisms distinct from clonal selection. Taken together, our work revealed an unappreciated role of the bone microenvironment in metastasis evolution and elucidated an epigenomic reprogramming process driving terminal-stage, multi-organ metastases.

INTRODUCTION

Metastasis to distant organs is the major cause of cancer-related deaths. Bone is the most frequent destination of metastasis in breast cancer and prostate cancer (Gundem et al., 2015; Kennecke et al., 2010; Smid et al., 2008). In the advanced stage, bone metastasis is driven by the paracrine crosstalk among cancer cells, osteoblasts, and osteoclasts, which together constitute an osteolytic vicious cycle (Esposito et al., 2018; Kang et al., 2003; Kingsley et al., 2007; Weilbaecher et al., 2011). Specifically, cancer cells secrete molecules such as PTHrP, which act on osteoblasts to modulate the expression of genes including RANKL and OPG (Boyce et al., 1999; Juárez and Guise, 2011). The alterations of these factors, in turn, boost osteoclast

maturation and accelerate bone resorption. Many growth factors (e.g., IGF1) deposited in the bone matrix are then released and reciprocally stimulate tumor growth. This knowledge laid the foundation for clinical management of bone metastases (Coleman et al., 2008).

The urgency of bone metastasis research is somewhat controversial. It has long been noticed that, at the terminal stage, breast cancer patients usually die of metastases in multiple organs. In fact, compared to metastases in other organs, bone metastases are relatively easier to manage. Patients with the skeleton as the only site of metastasis usually have better prognosis than those with visceral organs affected (Coleman and Rubens, 1987; Coleman et al., 1998). These facts argue that perhaps metastases in more vital organs should be prioritized in research. However,



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metastases usually do not occur synchronously. In 45% of metastatic breast cancer cases, bone is the first organ that shows signs of metastasis, much more frequently compared to the lungs (19%), liver (5%), and brain (2%) (Coleman and Rubens, 1987). More importantly, in more than two-thirds of cases, metastases will not be limited to the skeleton, but rather subsequently occur to other organs and eventually cause death (Coleman, 2006; Coleman and Rubens, 1987; Coleman et al., 1998). This raises the possibility of secondary dissemination from the initial bone lesions to other sites. Indeed, recent genomic analyses concluded that the majority of metastases result from seeding from other metastases, rather than primary tumors (Brown et al., 2017; Gundem et al., 2015; Ullah et al., 2018). Thus, it is imperative to investigate further metastatic seeding from bone lesions, as it might lead to prevention of the terminal stage, multi-organ metastases that ultimately cause the vast majority of deaths.

Despite its potential clinical relevance, little is known about metastasis-to-metastasis seeding. Current preclinical models focus on seeding from primary tumors but cannot distinguish between additional sites of dissemination. We have recently developed an approach, termed intra-iliac artery injection (IIA), that selectively deliver cancer cells to hind limb bones via the external iliac artery (Wang et al., 2015, 2018; Yu et al., 2016). Although it skips the early steps of the metastasis cascade, it focuses on the initial seeding of tumor cells in the hind limbs, and allows the tracking of secondary metastases from bone to other organs. It is therefore a suitable model to investigate the clinical and biological roles played by bone lesions in multi-organ metastasis-to-metastasis seeding.

RESULTS

Temporally lagged multi-organ metastases in mice carrying IIA-introduced bone lesions of breast and prostate cancers

IIA injection has been employed to investigate early-stage bone colonization. Both aggressive (e.g., MDA-MB-231) and relatively indolent (e.g., MCF7) breast cancer cells can colonize bones albeit following different kinetics. In both cases, cancer cell distribution is highly bone-specific at early time points, allowing us to dissect cancer-bone interactions without the confounding ef-

fects of tumor burden in other organs (Figure 1A) (Wang et al., 2015, 2018). However, as bone lesions progress, metastases, as indicated by bioluminescence signals, begin to appear in other organs, including additional bones, lung, liver, kidney, and brain, usually 4–8 weeks after IIA injection of MDA-MB-231 cells (Figure 1B). Bioluminescence imaging provides sufficient sensitivity to detect metastases (Deroose et al., 2007). However, many factors such as lesion depth and optical properties of tissues may influence signal penetration. Thus, we used a number of other approaches to validate the presence of metastases in multiple types of tissues. These include positron emission tomography (PET) (Figure 1C), micro computed tomography (μ CT) (Figures 1C and S1A), whole-tissue two-photon imaging (Figure S1B), immunofluorescence staining (Figure 1D and 1E), and histological staining (H&E) (Figure S1C). Compared to bioluminescence imaging, these approaches provided independent evidence, but are either less sensitive or non-quantitative (Deroose et al., 2007) (Figure S1A). Therefore, we also used quantitative PCR (qPCR) to detect human-specific DNA in dissected mouse tissues and confirmed that qPCR results and bioluminescence signal intensity values are highly correlative (Figures S1D and S1E). Of note, the spectrum of metastases covers multiple other bones (Figure 1D) and soft-tissue organs (Figure 1E). Taken together, our data support occurrence of multi-organ metastases in animals with IIA-introduced bone lesions.

This phenomenon is not specific for the highly invasive MDA-MB-231 cells, but was also observed in more indolent MCF7 cells and PC3 prostate cancer cells, as well as murine mammary carcinoma AT-3 cells in immunocompetent mice, albeit after a longer lag period for PC3 cells (8–12 weeks) (Figures 1F–1H and S1F).

As an independent approach to introduce bone lesions, we used intra-femoral (IF) injection that delivers cancer cells directly to bone marrow, bypassing the artery circulation involved in IIA injection. This approach also resulted in multi-organ metastases at late time points in both MDA-MB-231 and AT-3 models (Figures 1I and S1G). The frequency and distribution patterns of metastases were similar between intra-femoral and IIA injection models (Figures 1J and 1K). Thus, we hypothesize cancer cells in the bone microenvironment may gain capacity to further metastasize.

Figure 1. Multi-organ metastases in mice with bone lesions

(A) Diagram of intra-iliac artery (IIA) injection and representative bioluminescent images (BLI) showing the *in vivo* distribution of tumor cells after IIA injection of 1E5 MDA-MB-231 fLuc-mRFP cells.
(B and C) Representative *ex vivo* BLI images (B) and PET- μ CT (C) on hindlimb and other tissues of the same animal with MDA-MB-231 cells inoculated in the right hindlimb after 8 weeks. R.H, right hindlimb; Lu, lung; L.H, left hindlimb; Li, liver; Ki, kidney; Sp, spleen; Br, brain; Ve, vertebrae; F.L, forelimbs; Ri, ribs; St, sternum; Cr, cranium.
(D and E) Representative immunofluorescent images of tumor lesions in various bones (D) and other organs (E). To obtain complete views of entire organs, smaller fields were acquired in tiles by mosaic scanning and then stitched by Zen. Scale bar, 20 μ m.
(F–H) Representative BLI images of animals and tissues after IIA injection of 2E5 prostate cancer cells PC3 (F), 1E5 ER⁺ breast cancer cells MCF7 (G), and 1E5 murine mammary carcinoma cells AT-3 (H) at the indicated time.
(I) Diagram of intra-femoral injection (IF) (left) and representative *ex vivo* BLI images of tissues from animals received 1E5 MDA-MB-231 cells (middle) or AT-3 cells (right) via IF injection.
(J and K) Heatmap of *ex vivo* BLI intensity and status of metastatic involvement on various types of tissues from animals carried MDA-MB-231 (J) and AT-3 (K) bone tumors. Columns, individual animal; rows, various tissues or status of multi-site metastases; Gray, no detectable lesion. n (# of mice): MDA-MB-231, 16 (IIA), 11 (IF); AT-3, 10 (IIA), 10 (IF). p values were assessed by Fisher's exact test on the ratio of metastasis while by Mann-Whitney test on the tumor burden. See also Figure S1.

Bone lesions more readily give rise to multi-organ metastasis

The later-appearing multi-organ metastases may result from further dissemination of cancer cells in the initial bone lesions. Alternatively, they could also arise from cancer cells that leaked and escaped from bone capillaries during IIA or IF injection. In the latter case, the leaked cancer cells would enter the iliac vein and subsequently arrive in the lung capillaries. Indeed, there did appear to be bioluminescence signals in the lungs upon IIA injection (Figure 1A). To distinguish these probabilities, we performed intra-iliac vein (IIV) injection and compared the results to those of IIA injection at late time points. The IIV injection procedure should mimic the “leakage” from IIA injection, although this would allow many more cells to enter the venous system and be arrested in the lung capillaries (Figures 2A and S2A–S2C, compared to Figure 1A). As another relevant comparison, we also examined metastasis from orthotopic tumors transplanted into mammary fat pad (MFP) (Figures 2B, S2A, S2B, and S2D). Furthermore, in the case of ER⁺ cells, recent studies suggest that intra-ductal injection provides a more “luminal” microenvironment and may promote spontaneous metastasis to other organs (Sflomos et al., 2016). As a result, specifically for MCF7 cells, the only ER⁺ cancer model used in our study, we also included mouse intra-ductal (MIND) injection as an additional model. In all experiments, we used total bioluminescence signal intensity to evaluate tumor burdens at hind limbs (IIA and IF), lungs (IIV), and mammary fat pads (MFP and MIND), respectively. We attempted to assess multi-organ metastasis when the “primary lesions” reach a comparable level of bioluminescent intensity, simply to rule out the source tumor burden as a confounding factor in our comparisons. This was feasible for some models such as mammary tumors and bone lesions derived from MCF7 (Figure S2E). However, in other models, mammary tumors tend to grow much faster compared to lesions growing in other sites (Figures S2F and S2G). Therefore, we chose to end experiments at the same time point for all conditions. In all experiments, multi-organ metastases were examined well before animals became moribund. Taken together, we asked if secondary metastasis from bone lesions follows a faster kinetics and reaches a wider spectrum of target organs as compared to that from orthotopic tumors or lungs.

Strikingly, the answer to this question is evidently positive in all three tumor models examined (Figures 2C–2H). We assessed 11 organs including six other bones and five soft tissue organs for metastasis. Curiously, in many cases, counter-lateral hind limbs (designated as “L.Hindlimb” for “left hind limb” as the initial bone lesions were introduced to the right hind limb) are most frequently affected among all organs in IIA models. Lungs are also frequently affected in MDA-MB-231 and AT-3 models, by metastasis from both bone lesions and orthotopic tumors. However, it is striking to note that lung metastasis in IIA and IF models is comparable or even more severe as compared to that in IIV models, despite the fact that IIV injection delivers more cancer cells directly to lungs (Figure S2H). In fact, the normalized increase of tumor burden at lungs through IIA and IF are at least 10-fold more than that through IIV injection (e.g., Figure S2H), which strongly argue that bone microenvironment promotes secondary metastasis.

Cross-seeding of cancer cells from bone lesions to orthotopic tumors

Cancer cells may enter circulation and seed other tumor lesions or re-seed the original tumors (Kim et al., 2009). By using MDA-MB-231 cells tagged with different fluorescent proteins, we asked if bone lesions can cross-seed mammary tumors (Figure 3A). Interestingly, we observed that although orthotopic tumors can be readily seeded by cells derived from bone lesions, the reverse seeding rarely occurs (Figures 3B and 3C). This difference again highlights the enhanced metastatic aggressiveness of cancer cells in the bone microenvironment.

Parabiosis models support enhanced capacity of cancer cells to metastasize from bone to other organs

It is possible that IIA injection disturbs bone marrow and stimulates systemic effects that allow multi-organ metastases. For example, the injection might cause a transient efflux of bone marrow cells that can arrive at the distant organ to form pre-metastatic niche. To test this possibility, we used parabiosis to fuse the circulation between a bone lesion-carrying mouse (donor) and tumor-free mouse (recipient) 1 week after IIA injection. In parallel, we also performed parabiosis on donors that have received MFP injection and tumor-free recipients (Figure 3D). After 7 weeks, surgical separation was performed to allow time for metastasis development in the recipients. Subsequently, the organs of originally tumor-free recipients were collected and examined for metastases 4 months later. Only ~20% of recipients in the IIA group were found to harbor cancer cells in various organs (Figures 3E and 3F), mostly as microscopic disseminated tumor cells (Figure 3G), indicating that the fusion of circulation system is not efficient for metastatic seeds to cross over from donor to recipient. However, in the MFP comparison group, no metastatic cells were detected (Figures 3F, S3A, and S3B), and the difference is statistically significant. Therefore, the parabiosis data also support the hypothesis that the bone microenvironment invigorates further metastasis, and this effect is unlikely to be due to IIA injection-related systemic influence.

An evolving barcode system revealed the phylogenetic relationships between initial bone lesions and secondary metastases

Barcoding has become widely used to elucidate clonal evolution in tumor progression and therapies. An evolving barcoding system has recently been invented for multiple parallel lineage tracing (Kalhor et al., 2017, 2018). It is based on CRISPR/Cas9 system but utilizes guide RNAs that are adjacent to specific protospacer adjacent motif (PAM) in their genomic locus, thereby allowing Cas9 to mutate its own guide RNAs. These variant guide RNAs are named homing guide RNAs (hgRNAs). When Cas9 is inducibly expressed, hgRNA sequences will randomly drift, serving as evolving barcodes (Figure 4A). A preliminary *in vitro* experiment demonstrated that the diversity of barcodes (measured as Shannon entropy) is a function of duration of Cas9 expression (Figures 4B and S4A).

We introduced this system into MDA-MB-231 and AT-3 cells, and transplanted them into mammary fat pads of nude and wild-type C57BL/6 mice, respectively. When orthotopic tumors

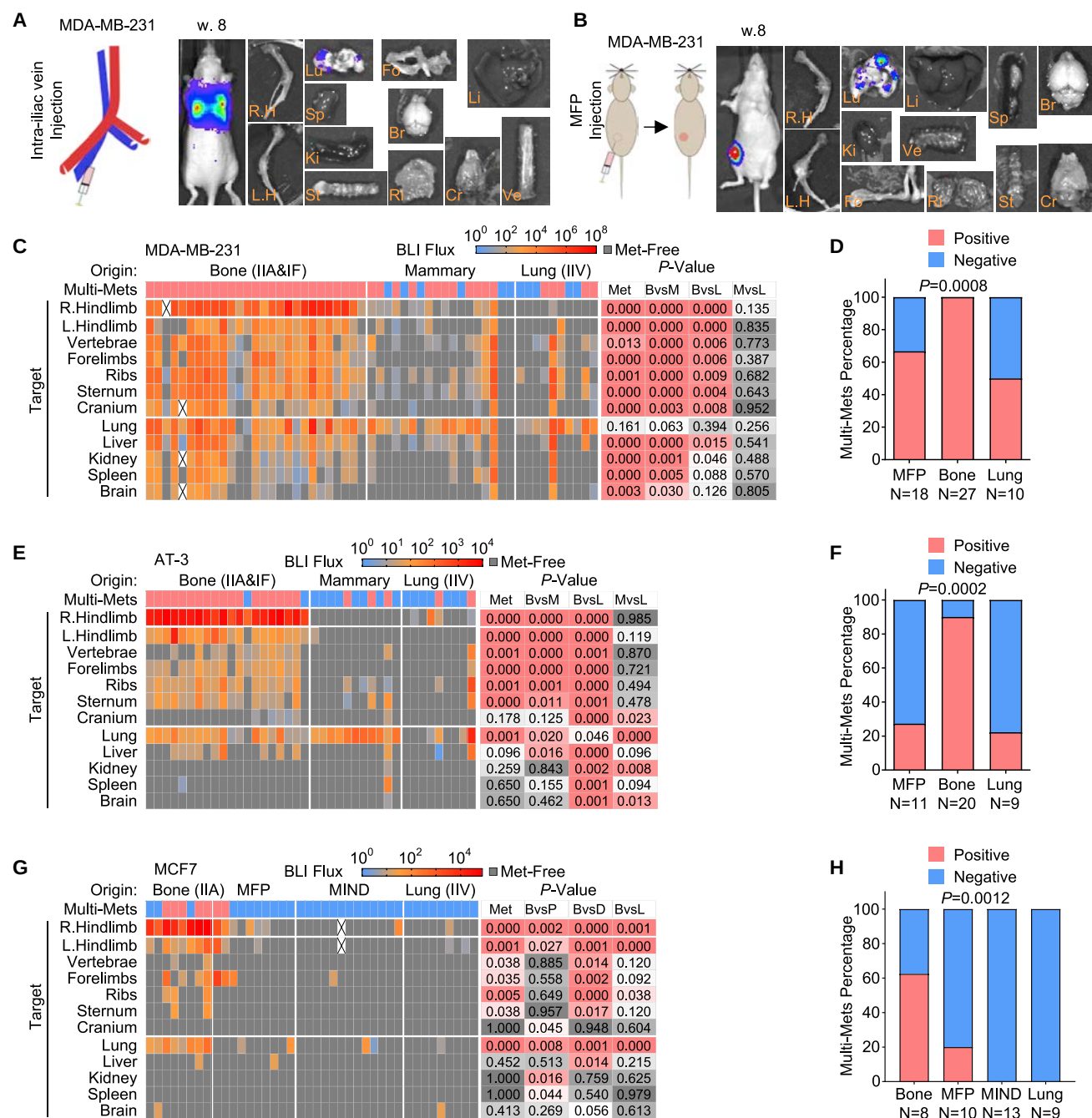


Figure 2. Bone microenvironment promotes further metastasis

(A) Diagram of intra-iliac vein (IIV) injection and representative BLI images of animals and tissues 8 weeks after IIV injection of 1E5 MDA-MB-231 cells.

(B) Diagram of mammary fat pad (MFP) implantation and representative BLI images of animals and tissues 8 weeks after MFP implantation of 1E5 MDA-MB-231 cells.

(C and D) Comparison of metastatic pattern and tumor burden (C) and the ratio of multi-site metastasis (D) in animals with bone (IIA/IF), lung (IIV) or mammary (MFP) tumors of MDA-MB-231 cells. n (# of mice) = 27 (bone); 18 (MFP); 10 (lung).

(E and F) Comparison of metastatic pattern and tumor burden (E) and the ratio of multi-site metastasis (F) in animals with bone (IIA/IF), lung (IIV) or mammary (MFP) tumors of AT-3 cells. n (# of mice) = 20 (bone); 11 (MFP); 9 (lung).

(G and H) Comparison of metastatic pattern and tumor burden (G) and the ratio of multi-site metastasis (H) in animals with bone (IIA), lung (IIV) or mammary (MFP or MIND) tumors of MCF7 cells. n (# of mice) = 8 (bone); 10 (MFP); 13 (MIND); 9 (lung).

p values were assessed by χ^2 test in (C)–(H) on the ratio of metastasis; by uncorrected Dunn's test following Kruskal-Wallis test in (C), (E), and (H) on the tumor burden.

See also Figure S2.

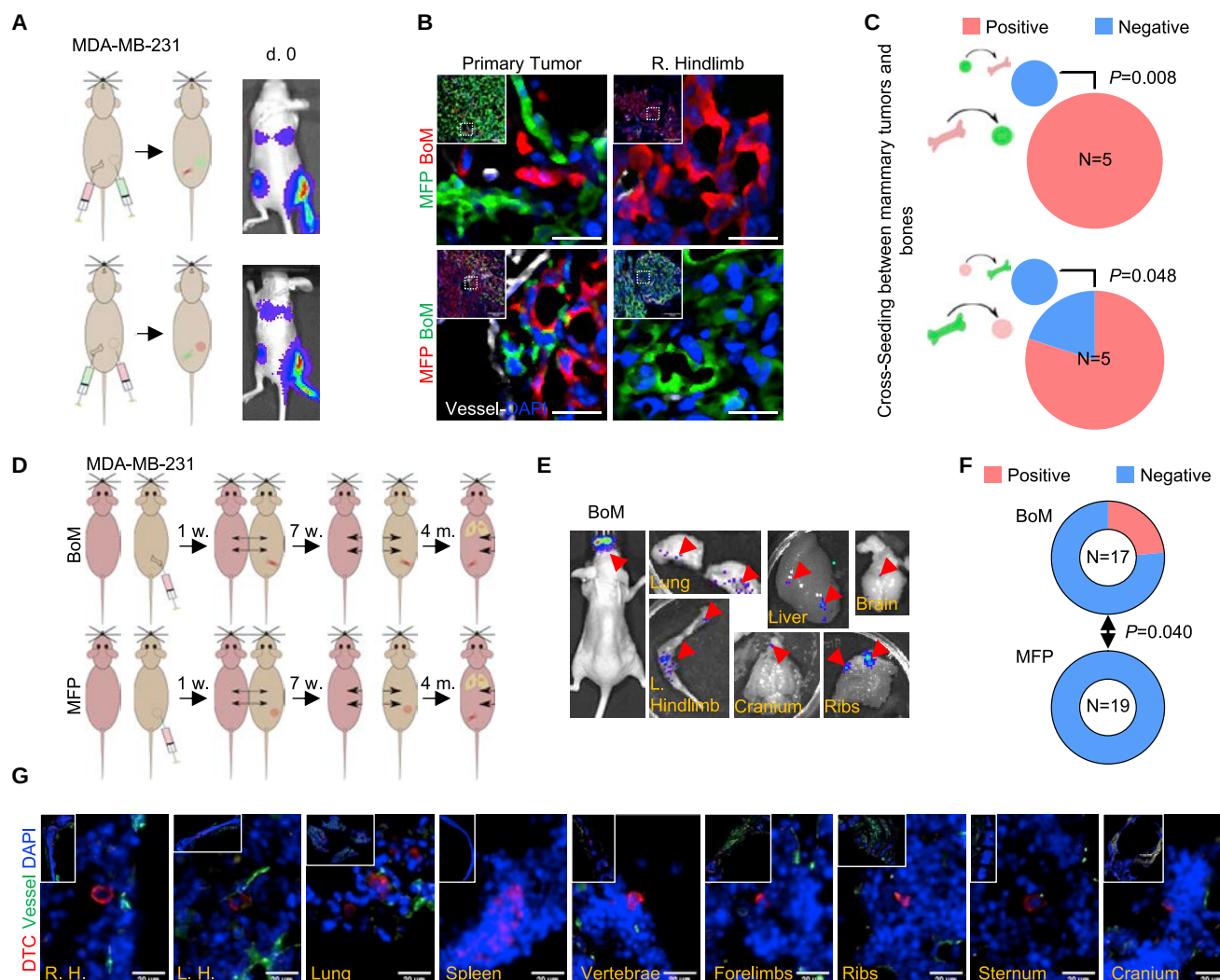


Figure 3. Cross-seeding and parabiosis experiments support the promoting effects of bone microenvironment on further dissemination
 (A) Experimental design of cross-seeding experiment between mammary and bone tumors of mRFP or EGFP tagged MDA-MB-231 cells. Upper: mRFP (IIA), EGFP (MFP). Lower: EGFP (IIA), mRFP (MFP).
 (B) Representative confocal images showing the cross-seeding between bone and mammary tumors. Scale bar, 20 μ m. n (# of mice) = 5 for each arm.
 (C) Incidence of cross-seeding between bone and mammary tumors.
 (D) Experimental design of parabiosis models to compare the metastatic capacity of bone and mammary tumors. n (# of mice) = 17 (BoM); 19 (MFP).
 (E) Representative BLI images of metastatic lesions in recipient mice parabiotic with mice bearing bone metastases.
 (F) Ratio of recipients with metastasis in bone and mammary tumor groups, as determined by BLI imaging.
 (G) Representative immunofluorescent images on tissues from recipients of bone tumor group. To obtain complete views of entire organs, smaller fields were acquired in tiles by mosaic scanning and then stitched by Zen. Scale bar, 20 μ m. Tissues from 6 animals were examined.
 p values were assessed by Fisher's exact test in (C) and (F).
 See also Figure S3.

reach 1 cm³, we resected the tumors and induced Cas9 by doxycycline. It should be noted that the orthotopic tumors already harbored a high diversity of mutant barcodes presumably due to leakage of Cas9 expression. This served as an initial barcode repertoire that enabled us to distinguish distinct clones that metastasize from orthotopic tumors to various organs. Further Cas9 expression yielded new mutations for delineation of parent-child relationship among lesions (Figure 4C). We rationalize that the diversity of barcodes, or the Shannon entropy,

in a metastasis should reflect the “age” of metastasis. When secondary metastasis occurs, child metastases will inherit only a subset of barcodes causing a reduction of Shannon entropy. Therefore, among genetically related metastases indicated by sharing common mutant barcodes, those with higher Shannon entropy are more likely to be parental (Figure 4C). This can be supported by the observation that primary bone lesions possess higher entropy than those secondary metastases in IIA model (Figure S4B).

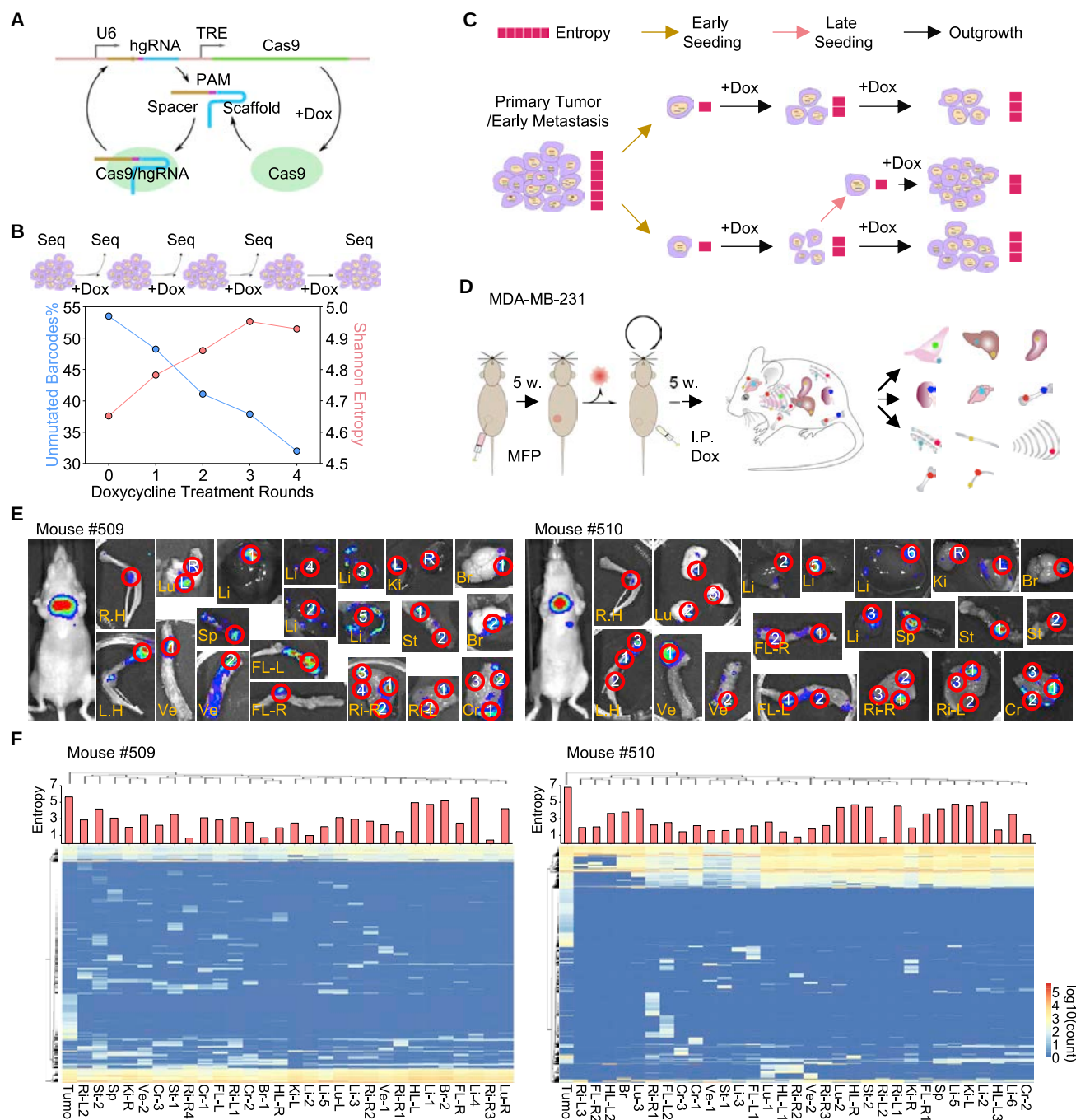


Figure 4. In vivo barcoding of spontaneous metastases with hgRNAs

(A) Principle of the evolving barcode system comprised of hgRNAs and inducible Cas9.

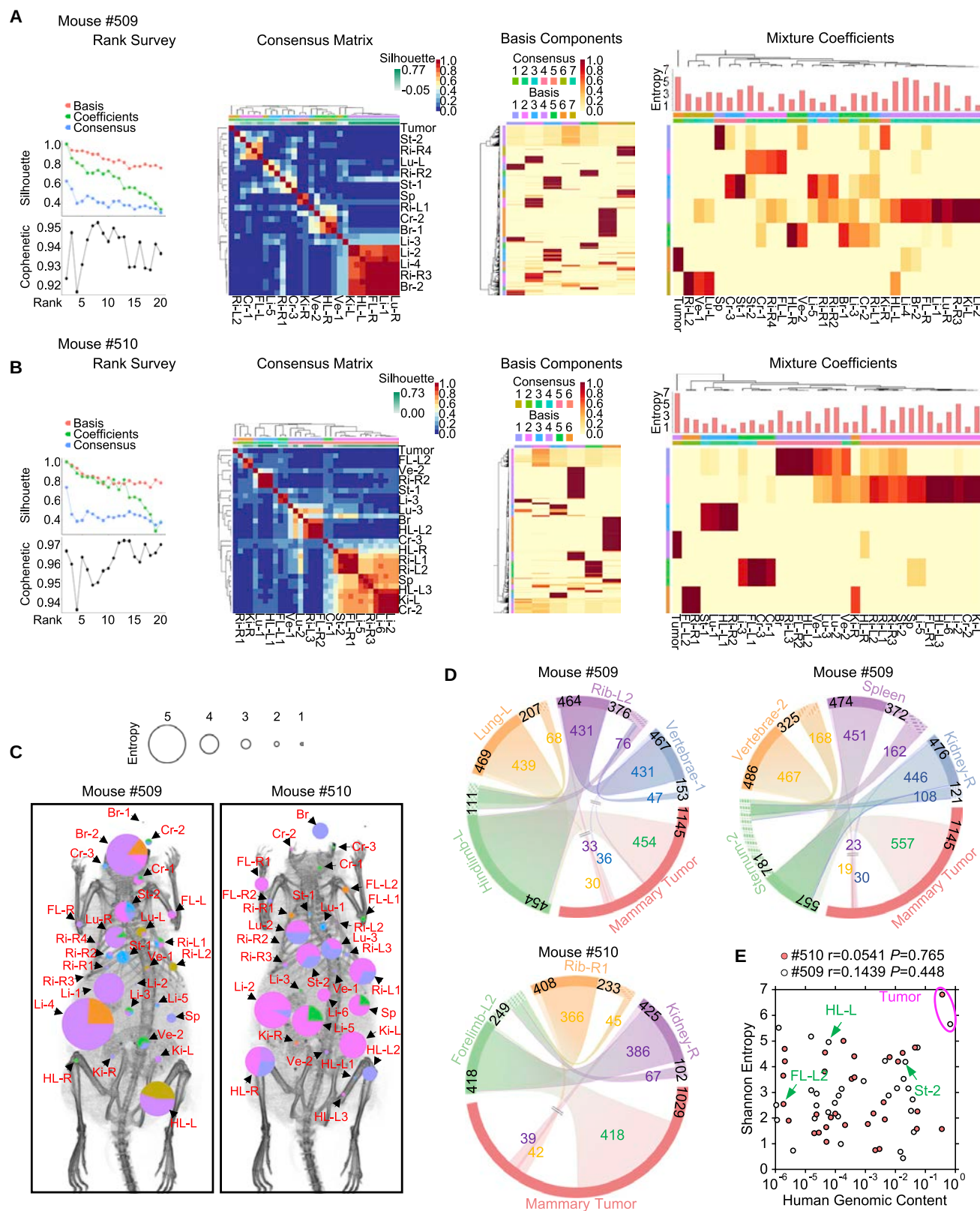
(B) Ratio of unmutated barcode and Shannon entropy in MDA-MB-231 cells upon multiple rounds of doxycycline treatment *in vitro*.

(C) Schematics showing the rationale of using evolving barcodes to infer the evolution of metastatic lesions and the timing of seeding events. Barcode diversity decreases during the seeding process. Children metastases inherit a subset of signature barcodes from parental tumors. Upon Cas9 activation, barcodes start evolving and regain diversity. Diversity of barcodes can therefore infer the relative timing of seeding, and phylogenetically related metastases share a subset of signature barcodes.

(D and E) Design of *in vivo* barcoding experiment (D) and representative BLI images (E) of metastatic lesions from MDA-MB-231 tumors.

(F) Feature matrix of mutation events in MDA-MB-231 metastatic samples.

See also Figure S4 and Table S1.



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We isolated 29, 32, 9, and 17 metastases from two mice bearing MDA-MB-231 and two mice bearing AT3 tumors, respectively (Figures 4D, 4E, and S4C; Table S1). Sequencing of the barcodes carried by these metastases in combination of the analysis of the timing of seeding as indicated by the Shannon entropy of barcodes led to profound findings. First, in line with a previous study (Echeverría et al., 2018), multi-organ metastases are not genetically grouped according to sites of metastases at the terminal stage (Figures 4F and S4D). Nonnegative matrix factorization (NMF) analysis of mutant barcodes suggested the early disseminated metastases, which have the highest level of Shannon entropy, were featured with a common cluster of mutant barcodes irrespective of their locations, especially in AT-3 models (Figures 5A–5C and S5A–S5C). This is evidence against organotropism in the late stage of metastatic progression in mouse models. Second, most metastases are potentially multiclonal as indicated by multiple clusters of independent mutant barcodes (Figures 5C and S5C). Third, putative parent-child relationship between metastases with unique mutant barcodes clearly exemplified secondary metastatic seeding from bone to other distant sites (Figures 5D and S5D) in both models. Finally, we did not observe a clear correlation between tumor burden and Shannon entropy across different metastases, and the putative funder metastases can be small in tumor burden while diversified in barcode composition, suggesting that asymptomatic metastases might also seed further metastases (Figures 5E and S5E). Taken together, these data reveal potential widespread metastasis-to-metastasis seeding and support that secondary metastases from the bone to other distant organs happen in a natural metastatic cascade.

The bone microenvironment promotes further metastasis by enhancing cancer cell stemness and plasticity

Organotropism is an important feature of metastasis. Clonal selection appears to play an important role in organ-specific metastasis, which has been intensively studied (Bos et al., 2009; Kang et al., 2003; Minn et al., 2005; Vanharanta and Massagué, 2013). Here, the metastasis-promoting effects of the bone microenvironment appear to be multi-organ and do not show specific organ-tropism. In an accompanied study, we discovered profound phenotypic shift of ER⁺ breast cancer cells in the bone microenvironment, which included loss of luminal features and gain of stem cell-like properties (Bado et al., 2021). This shift is expected to promote further metastases (Gupta et al., 2019; Ye and Weinberg, 2015). Therefore, we hypothesize that the enhancement of metastasis may be partly through an epigenomic dedifferentiation process.

To test this possibility, we compared the metastasis capacity of a genetically identical SCP of MDA-MB-231 cells and its derivatives entrained by different microenvironments (Figures S6A and S6B). Based on a previous study (Minn et al., 2005), we picked a non-metastatic SCP termed SCP21. SCP21 cells were introduced to mammary fat pads, lungs, and hind limb bones to establish tumors. After 6 weeks, entrained cancer cells were extracted from these organs for further experiments (Figure 6A). We used intra-cardiac injection to simultaneously deliver cancer cells to multiple organs (Figure 6A). Compared to the mammary fat pad- and lung-entrained counterparts, bone-entrained SCP21 was more capable of colonizing distant organs and gave rise to much higher tumor burden in multiple sites as determined by bioluminescence (Figures 6A–6C). In mice subjected to dissection and *ex vivo* bioluminescent imaging, significantly more and bigger lesions were observed from mice received bone-entrained SCP21 cells in both skeletal and visceral tissues (Figures S6C–S6E), suggesting an increase of overall metastatic capacity rather than bone tropism in tumor cells exposed to bone environment.

Inspired by the accompanied study (Bado et al., 2021), we examined stemness markers (Al-Hajj et al., 2003; Charafe-Jauffret et al., 2009) of SCP21 cells entrained in different microenvironments. Interestingly, bone-entrained cells appeared to express a higher level of both ALDH1 activity and CD44 expression (Figures 6D and 6E). In addition, bone-entrained SCP21 cells increased expression of multiple proteins involved in epithelial-mesenchymal transition (EMT) and in pathways shown to mediate the effects of bone microenvironment on ER⁺ cancer cells in our accompanied study, including FGFR1, PDGFR β , EZH2, SLUG, and ZEB1 (Figures 6F and S6F). These data suggest that similar mechanisms may be at work to induce cancer cell stemness and plasticity in this ER⁺ model.

Indeed, when the same approaches were applied to the SCP2 derivatives of MCF7 cells. Bone entrained MCF7-SCP2 cells showed increased initial survival and faster metastatic growth after intra-cardiac injection (Figures 6G–6I) and increased level of ALDH1 activity and CD44 expression (Figures 6J and 6K). In this epithelial model, we also observed a hybrid EMT phenotype (Figure S6G), as also elaborated in our associated study (Bado et al., 2021). It should be noted that, in this series of experiments, lung-derived subline was not developed due to the lack of lung colonization for MCF7-SCP2 cells.

In addition to cancer cells that are manually extracted from various organs, we examined naturally occurred circulating tumor cells (CTCs) emitted from bone lesions versus mammary tumors. Not surprisingly, bone lesions generated a higher number of CTCs, probably due to the more permeable vascular structures or survival advantage conferred by the bone.

Figure 5. NMF analysis of evolving barcodes delineates metastatic spread

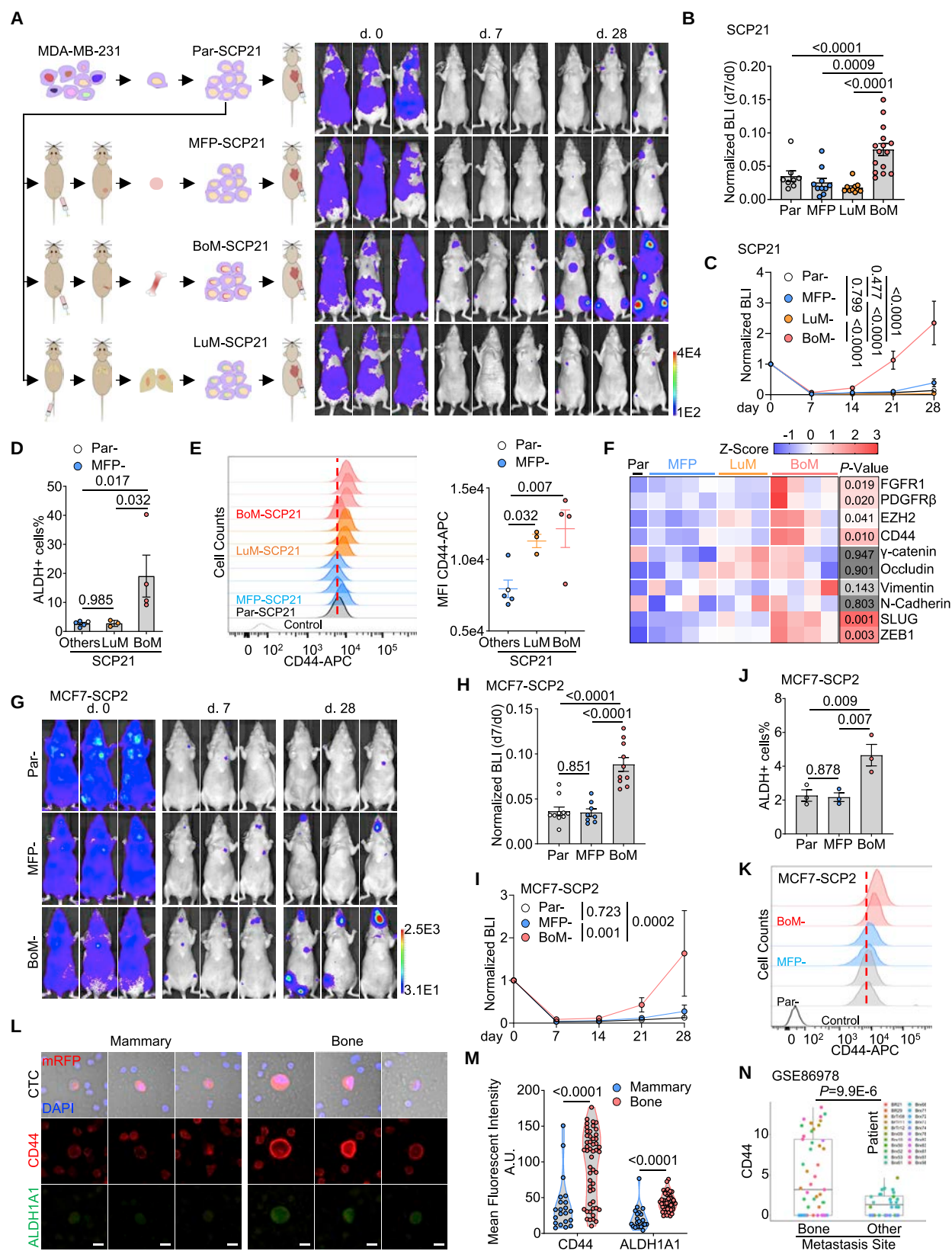
(A and B) Plots of NMF rank survey, consensus matrix, basis components matrix and mixture coefficients matrix of 200 NMF runs on the barcodes from MDA-MB-231 metastatic lesions.

(C) Body map depicting the basis composition of MDA-MB-231 metastatic lesions.

(D) Chord diagrams illustrating the composition flow of barcode mutations between primary tumors and selected MDA-MB-231 metastatic lesions.

(E) Correlation plot of Shannon entropy and tumor burden on MDA-MB-231 samples. The tumor burden was indicated by human genomic content determined by q-PCR. Spearman r and p values were indicated.

See also Figure S5.



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However, on top of the higher number, CTCs from bone lesions also express higher quantity of CD44 and ALDH1 (Figures 6L and 6M), suggesting increased stemness.

Finally, we also interrogated CD44 expression in a single-cell RNA sequencing (RNA-seq) dataset of CTCs derived from breast cancer patients. When patients were divided into two groups—those carrying bone metastasis versus those carrying other metastases, significantly higher expression of CD44 was observed in the former (Figure 6N) (Aceto et al., 2018), providing clinical support for our hypothesis that the bone microenvironment promotes tumor cell stemness and plasticity, and thereby invigorate further metastasis.

EZH2 in cancer cells orchestrates the effect of bone microenvironment in secondary metastasis

Because EZH2 was revealed to play a central role in loss of ER and gain of plasticity in ER⁺ models in the accompanied study (Bado et al., 2021), we asked if it also mediates secondary metastasis. The frequency of ALDH1⁺ cells and the expression of mesenchymal and stemness markers in bone-entrained SCP21 cells were also significantly decreased upon treatment of an EZH2 inhibitor (EPZ) used in our accompanied study (Figures S7A–S7C), supporting EZH2 as an upstream regulator the observed phenotypic shift.

In addition to expression, we used an EZH2 target gene signature (Lu et al., 2010) to deduce EZH2 activities. This signature was then applied to RNA-seq transcriptomic profiling of SCP21 cells subjected to various treatments or entrained in different organs. EPZ treatment relieved the suppression of signature genes, resulting in higher expression (Figure S7D), which supported the validity of the signature. We then compared cells entrained in bone lesions versus mammary gland tumors or lung metastasis and observed significantly higher EZH2 activity in the bone-entrained cells (Figure 7A). Importantly, bone-induced changes to both EZH2 activity and frequency of ALDH1⁺ cells appeared to be reversible, as *in vitro* passages led to progressive loss of these traits (Figures 7B and 7C). Other bone microenvironment-induced factors upstream of EZH2 (e.g., FGFR1 and PDGFR β) (Kottakis et al., 2011; Yue et al., 2019) also exhibited transient increased expression in bone-entrained cells (Figures 7D and

S7E). Taken together, these data further suggest the potential role of EZH2 in secondary metastasis from bone lesions.

Remarkably, transient treatment of EPZ before intra-cardiac injection, which did not suppress the growth of tumor cells *in vitro* (Figure S7F), completely abolished the enhanced metastasis of bone-entrained SCP21 cells (Figures 7E–7G) and MCF7-SCP2 cells (Figures 7H–7K) *in vivo*, again demonstrating that the observed effects of bone microenvironment is not through clonal selection, but rather epigenomic reprogramming driven by EZH2.

Finally, to confirm the cancer cell-intrinsic role of EZH2 during this process, we generated inducible knockdown of EZH2 (Figure S7G), that also slightly affected downstream expression of plasticity factors and stem cell markers (Figures S7G and S7H), but did not alter cancer cell growth rate *in vitro* (Figure S7I). Induction of knockdown was initiated after bone lesions were introduced for one week (Figures 7L, and S7J). Interestingly, whereas EZH2 knockdown did not alter primary bone lesion development (Figure 7M), it dramatically reduced secondary metastasis to other organs (Figure 7N). Taken together, these aforementioned results strongly implicate EZH2 as a master regulator of secondary metastases from bone lesions.

DISCUSSION

In this study, based on the IIA injection technique and through multiple independent approaches, we demonstrated that the bone microenvironment not only permits cancer cells to further disseminate but also appears to augment this process. A key question that remains is the timing of secondary metastasis spread out of the initial bone lesions: whether this occurs before or after the bone lesions become symptomatic and clinically detectable. The answer will determine if therapeutic interventions should be implemented in adjuvant or metastatic settings, respectively. Moreover, if further seeding occurs before bone lesions become overt, it raises the possibility that metastases in other organs might arise from asymptomatic bone metastases, which might warrant further investigations. Indeed, it has been reported that DTCs in bone marrow of early breast cancer patients enrich stem cell-like population (Alix-Panabieres et al.,

Figure 6. Bone-entraining boosts the metastatic capacity of single cell-derived cancer cells

(A) Experimental design (left) and representative BLI images (right) to test the metastatic capacity of mammary, lung, or bone-entrained SCP21s.
(B) Normalized whole-body BLI intensity 7 days after intra-cardiac (IC) injection of same number of MFP-, LuM-, BoM-, or Par-SCP21 cells.
(C) Colonization kinetics of MFP-, LuM-, BoM-, and Par-SCP21 cells after IC injection. n (# of mice) = 8 (Par); 10 (MFP); 15 (BoM); 10 (LuM).
(D) Percentage of ALDH⁺ population in MFP-, LuM-, BoM-, and Par-SCP21 cells by flow cytometry.
(E) Histogram (left) and median fluorescent intensity (MFI) (right) of surface CD44 protein in MFP-, LuM-, BoM- and Par-SCP21 cells by flow cytometry.
(F) Expression levels of proteins in Par- and organ-entrained SCP21s. Protein levels were quantified and converted into Z score from three or four western blottings.
(G–I) Representative BLI images (G), normalized BLI intensity at day 7 (H), and the colonization kinetics (I) of MFP-, BoM-, and Par- MCF7-SCP2 cells after I.C. injection. n (# of mice) = 10 (Par); 8 (MFP); 10 (BoM).
(J and K) Percentage of ALDH⁺ population (J) and expression of surface CD44 (K) in MFP-, BoM-, and Par- MCF7-SCP2 cells by flow cytometry. n (# of repeats) = 3 (J); 2 (K).
(L and M) Representative fluorescent images (L) and quantification (M) of CD44 and ALDH1A1 expression on CTCs from NRG mice bearing MDA-MB-231 cells derived mammary or bone tumors. CTCs were pooled from 5 blood samples. Scale bar, 10 μ m.
(N) Expression levels of CD44 mRNA in CTCs from breast cancer patients with bone metastases or other metastases (GSE86978).
Data are represented as mean $\pm$ SEM in (B), (C), (D), (E), (H), (I) and (J). p values were assessed by Fisher's LSD test following one-way ANOVA test in (B), (D), (E), (H), and (J); by Fisher's LSD test post two-way ANOVA test in (C) and (I); by Student's t test in (F); by Mann-Whitney test in (M) and (N).
See also Figure S6.

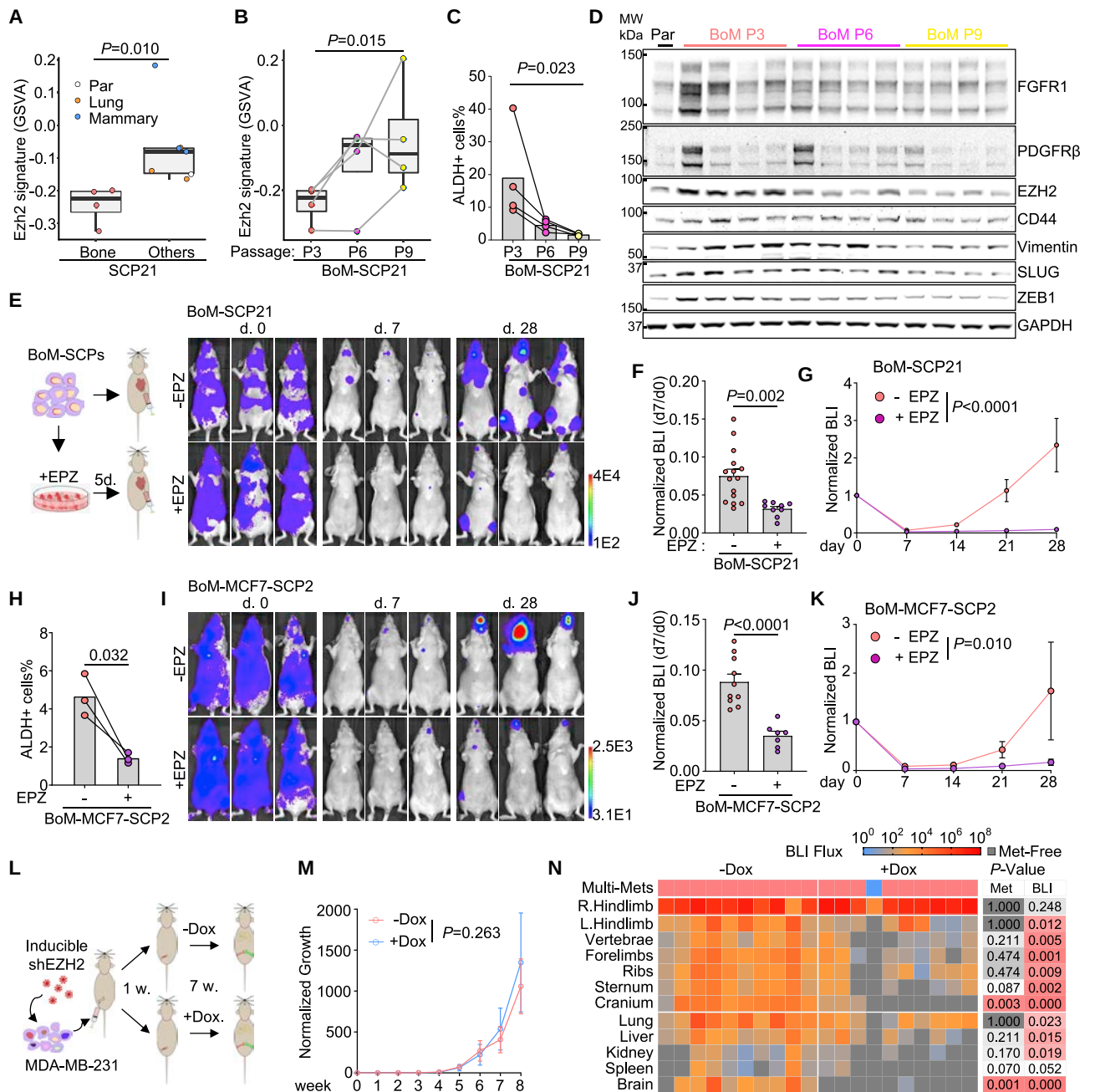


Figure 7. Secondary metastasis from bone lesions is dependent on EZH2 mediated epigenomic reprogramming

(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.
(E–G) The schematic diagram and representative BLI images (E), normalized BLI intensity at day 7 (F), and the colonization kinetics (G) of BoM-SCP21 cells with *in vitro* EPZ011989 (EPZ) treatment before IC injection. Non-treated BoM-SCP21 cells were used as control. n (# of mice) = 15 (–EPZ); 9 (+EPZ).
(H) Comparison of ALDH1⁺ cells in EPZ treated and non-treated BoM-MCF7-SCP2 cells by flow cytometry. n (# of replicate) = 3.
(I–K) Representative BLI images (I), normalized BLI intensity at day 7 (J), and the colonization kinetics (K) of BoM-MCF7-SCP2 cells with *in vitro* treatment of EPZ before IC injection. Non-treated BoM-MCF7-SCP2 cells were used as control. n (# of mice) = 10 (–EPZ); 7 (+EPZ).
(L) Experimental design assessing the multi-site metastases from bone lesions with inducible depletion of EZH2.
(M) Growth kinetics of the primary bone lesions in mice receiving doxycycline or control water, assessed by *in vivo* BLI imaging. BLI intensities at right hindlimbs were normalized to the mean intensity at day 0. n (# of mice) = 10 for each arm.

(legend continued on next page)

2007; Balic et al., 2006), supporting that asymptomatic bone micrometastases are potentially capable of metastasizing before being diagnosed. In this study, our evolving barcode strategy exemplified potential metastases from bone to other organs. Interestingly, we found that the putative parental metastases could remain small (Figures 5E and S5E), which may suggest that further dissemination might occur before diagnosis of existing lesions. Future studies will be needed to precisely determine the onset of secondary metastasis from bones.

The fact that the genetically homogeneous SCP cells became more metastatic after lodging into the bone microenvironment suggests a mechanism distinct from genetic selection. Remarkably, this phenotype persists even after *in vitro* expansion, so it is relatively stable and suggests an epigenomic reprogramming process. We propose that this epigenetic mechanism may act in concert with the genetic selection process. Specifically, the organ-specific metastatic traits may pre-exist in cancer cell populations (Minn et al., 2005; Zhang et al., 2013) and determine the first site of metastatic seeding. The epigenomic alterations will then occur once interactions with specific microenvironment niches are established and when cancer cells become exposed chronically to the foreign milieu of distant organs. Our data suggest that such alterations drive a second wave of metastases in a less organ-specific manner. This may explain why terminal stage of breast cancer is often associated with multiple metastases (Disibio and French, 2008).

Here, we suggested that the enhanced EZH2 activity underpins the epigenetic reprogramming of tumor cells in bone microenvironment for further metastases. EZH2 maintains the de-differentiated and stem cell-like status of breast cancer cells by repressing the lineage-specific transcriptional programs (Chang et al., 2011; Gonzalez et al., 2014). Pharmacologically or genetically targeting EZH2 has been reported to inhibit tumor growth, therapeutic resistance, and metastases with different efficacies in preclinical models (Hirukawa et al., 2018; Ku et al., 2017; Ma et al., 2020; Zhang et al., 2020). It was noted that in our models, EZH2 inhibition could not suppress the cell growth *in vitro* or in the primary injection site, whereas both the transient treatment of EZH2 inhibitor or inducible knockdown of EZH2 in cancer cells dramatically decreased secondary metastasis, suggesting targeting EZH2 may block the metastatic spread rather than the tumor growth.

In the clinic, some bone metastases can be managed for years without further progression, while others quickly develop therapeutic resistance and are associated with subsequent metastases in other organs (Coleman, 2006). These different behaviors may suggest different subtypes of cancers that are yet to be characterized and distinguished. Alternatively, there may be a transition between these phenotypes. In fact, depending on different interaction partners, the same cancer cells may exist in different status in the bone. For instance, although endothelial cells may keep cancer cells in dormancy (Ghajar et al., 2013;

Price et al., 2016), osteogenic cells promote their proliferation and progression toward micrometastases (Wang et al., 2015, 2018). Therefore, it is possible that the transition from indolent to aggressive behaviors is underpinned by an alteration of specific microenvironment niches. Detailed analyses of such alteration will lead to unprecedented insights into the metastatic progression.

Limitations the study

In this study, we did not provide direct clinical evidence that bone metastasis can seed other metastases in cancer patients. Although this is reported in several previous studies (Brown et al., 2017; Gudem et al., 2015; Ullah et al., 2018), the prevalence of secondary metastases seeded from bone lesions remains to be determined in patients. Future genomic studies with improved depth and informatics will likely dissect this process in greater depth and elucidate spatiotemporal paths of metastasis. The evolving barcode strategy was useful in tracing metastatic evolution. The most striking and robust finding from these experiments is that genetically closely related metastases do not localize in the same type of tissues and are usually highly distinct from orthotopic tumors. This in principle argues against independent seeding events from primary tumors and supports metastasis-to-metastasis seeding. However, the deduction of specific parental-child relationship based on Shannon entropy is intuitive and qualitative, and needs to be analyzed by more quantitative models in future work.

We principally demonstrated that compared to lungs, bones are more capable of promoting secondary metastasis. However, further studies will be required to determine whether other organs, such as liver and lymph nodes, might also exert similar effects on metastatic spread. We postulate that two factors may regulate an organ's capacity to promote secondary metastases. First, the initial wave of organotropic metastasis will pose a selection of metastatic seeds that arrive and survive in a specific organ. Second, the subsequent interactions with resident cells will induce adaptive epigenetic changes on top of the genetic selection, which together dictate whether the metastatic journey may extend to other organs.

Although data presented in this study indicate that cancer cells colonizing the bone acquire intrinsic traits for further dissemination, we cannot rule out systemic effects that may also contribute to this process. At the late stage, bone metastases are known to cause strong systemic abnormality such as cachexia (Waning et al., 2015), which may influence secondary metastasis. Even at early stages before bone metastases stimulate severe symptoms, the disturbance of micrometastases to hematopoietic cell niches may mobilize certain blood cells to migrate to distant organs, which may in turn result in altered metastatic behaviors (Peinado et al., 2017). These possibilities will need to be tested in future research.

(N) Heatmap of *ex vivo* BLI intensity and status of metastatic involvement in tissues from animals with EZH2 depleted or control bone metastases.

Data are represented as mean $\pm$ SEM in (F), (G), (J), (K), and (M). p values were assessed by Student's t test in (A), (F), and (J); by test for linear trend following repeat measure one-way ANOVA in (B) and (C); by LSD test following two-way ANOVA in (G), (K), and (M); by ratio paired t test in (H); by Fisher's exact test on the ratio of metastatic involvement and Mann-Whitney test on BLI intensity in (N).

See also Figure S7.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.cell.2021.03.011>.

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The authors declare no competing interests.

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Exploiting bone niches: progression of disseminated tumor cells to metastasis

Aaron M. Muscarella,^{1,2,3,4} Sergio Aguirre,^{1,2,3,4} Xiaoxin Hao,^{1,2,3} Sarah M. Waldvogel,^{1,2,3,5} and Xiang H.-F. Zhang^{1,2,3,6}

¹Lester and Sue Smith Breast Center, Baylor College of Medicine, Houston, Texas, USA. ²Dan L. Duncan Cancer Center, Baylor College of Medicine, Houston, Texas, USA. ³Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas, USA. ⁴Graduate Program in Integrative Molecular and Biomedical Sciences, Baylor College of Medicine, Houston, Texas, USA. ⁵Medical Scientist Training Program, Baylor College of Medicine, Houston, Texas, USA. ⁶McNair Medical Institute, Baylor College of Medicine, Houston, Texas, USA.

(1. AUTHOR: Please confirm that all author names are correct and complete.)

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
“predominantly occupy”. Is your meaning as intended?)** and 6
contribute to maintaining HSC quiescence by producing CXCL4 7
and TGF- β (69). 8

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
clarify. (b) Do all edits to this sentence express the correct
meaning?)** distinct cell differentiation routes, some of which 35
may skip intermediate steps. For instance, the platelet-producing 36
megakaryocytes can skip the intermediate oligopotent progenitors 37
and directly generate from megakaryocyte-primed HSCs (72, 73). 38
39

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

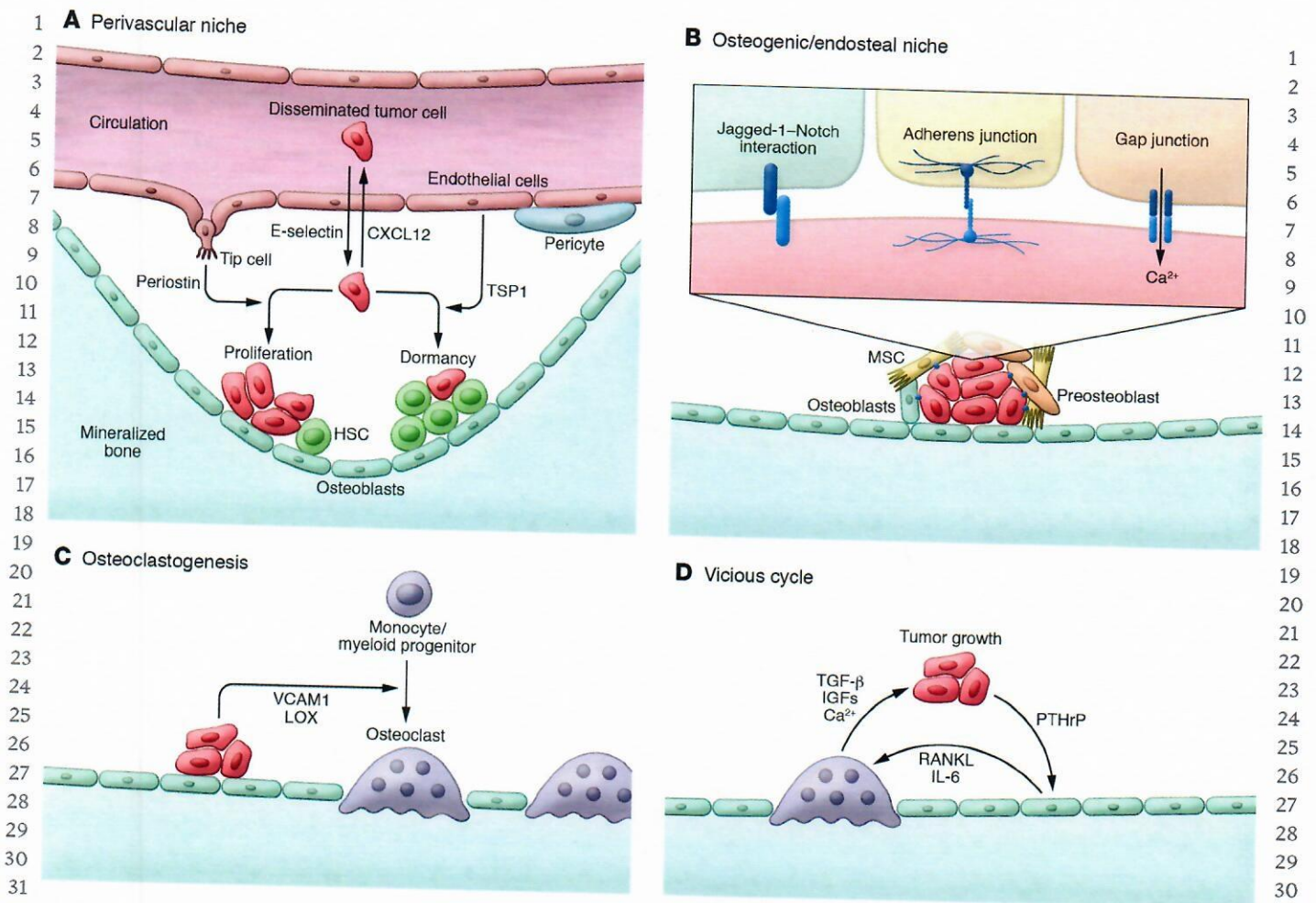


Figure 1. Disseminated tumor cells utilize bone marrow niches to survive and colonize. Disseminated tumor cells (DTCs) occupy many niches in the bone after they arrive in the organ via the bloodstream. When cells first extravasate from the vessels, they may reside in the perivascular niche. DTCs occupy 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 that act on tumor cells, further stimulating their growth. HSC, hematopoietic stem cell; LOX, lysyl oxidase; MSC, mesenchymal stem cell; PTHrP, 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 remod-
 eling 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).

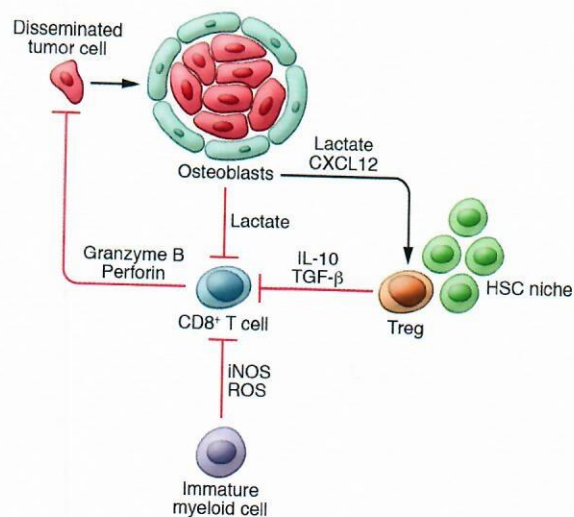


Figure 2. The immunosuppressive microenvironment in the bone and bone marrow. DTCs residing (22. **AUTHOR: Changed "reside" to "residing". Is your meaning as intended?**) in the bone marrow niches may produce many immunosuppressive factors that also interfere with cytotoxic CD8⁺ T cells. Among these 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

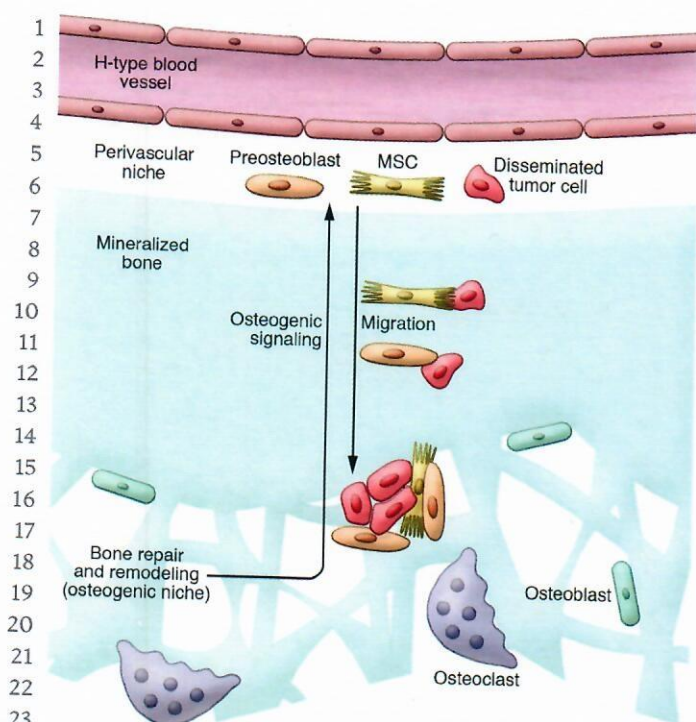


Figure 3. A potential connection between bone repair/remodeling and bone colonization of DTCs. One process that bridges different bone marrow niches is bone repair/remodeling. In this process, MSCs migrate from their perivascular origin to areas of bone repair or turnover. Recent evidence suggests that 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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Address correspondence to: Xiang H.-F. Zhang, Baylor College of Medicine, (20. **AUTHOR: Is “Baylor College of Medicine” correct as added to this mailing address?**) BCM600, One Baylor Plaza, Houston, Texas 77030, USA. Phone: 713.798.6239; Email: xiangz@bcm.edu.

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ARTICLE OPEN



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

¹Lester and Sue Smith Breast Center, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA. ²Dan L. Duncan Cancer Center, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA. ³Department of Molecular and Cellular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA. ⁴Graduate Program in Integrative Molecular and Biomedical Sciences, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA. ⁵Department of Cell Biology and Neuroscience, Institute for Quantitative Biomedicine, Rutgers University, 174 Frelinghuysen Road, Piscataway, NJ 08854, USA. ⁶Department of Biochemistry and Molecular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA. ⁷National Center for Macromolecular Imaging, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA. ⁸Department of Bioengineering, Stanford University, Stanford, CA 94305, USA. ⁹McNair Medical Institute, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA.

email: xiangz@bcm.edu

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

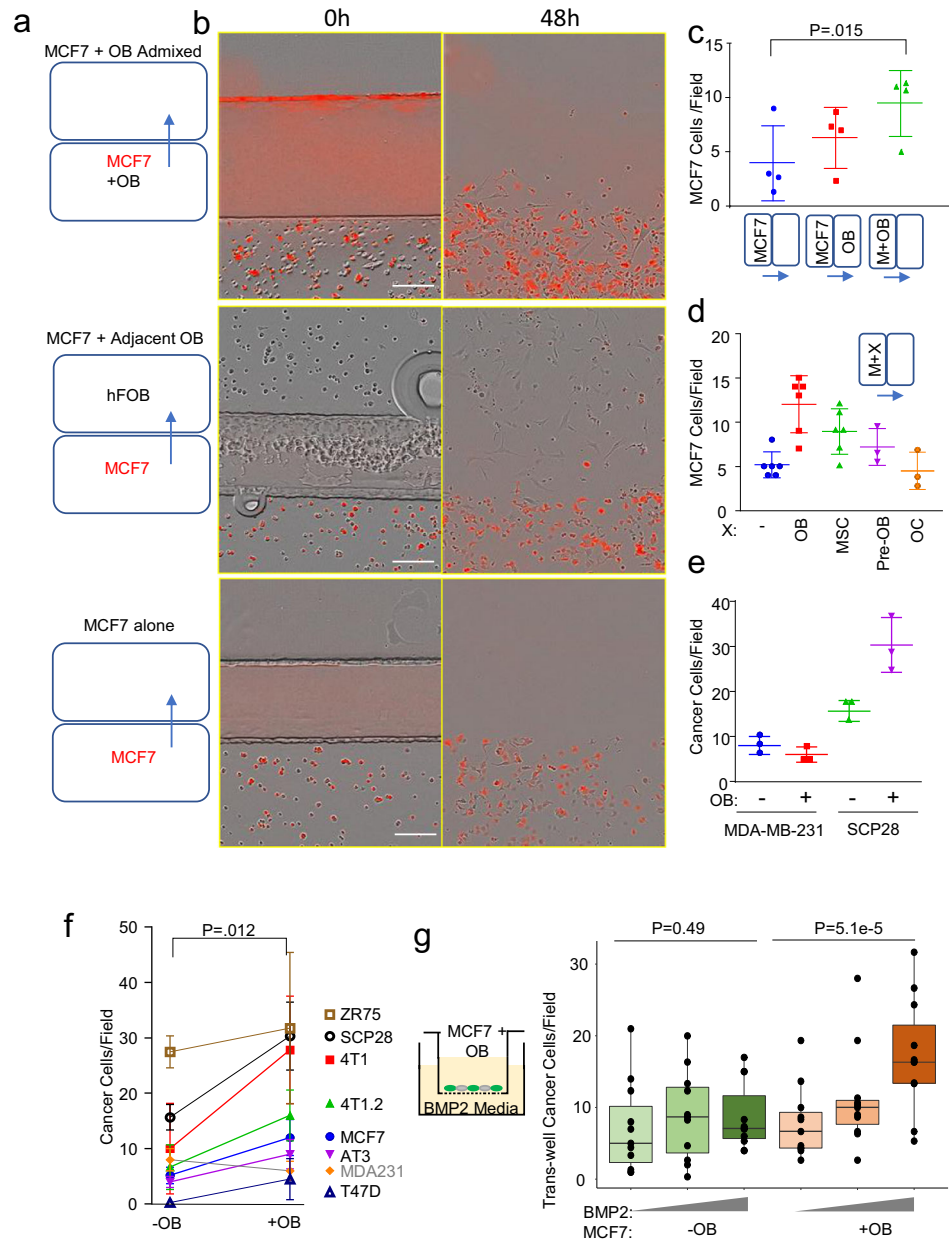


Fig. 1 Breast cancer cells which express E-cadherin have increased migration when cultured with osteogenic cells in vitro. **a** Ibidi silicone removable chambers were used to quantify migration by breast cancer cells in monoculture, in co-culture with osteoblasts in adjacent chambers, or in direct admixture 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”.

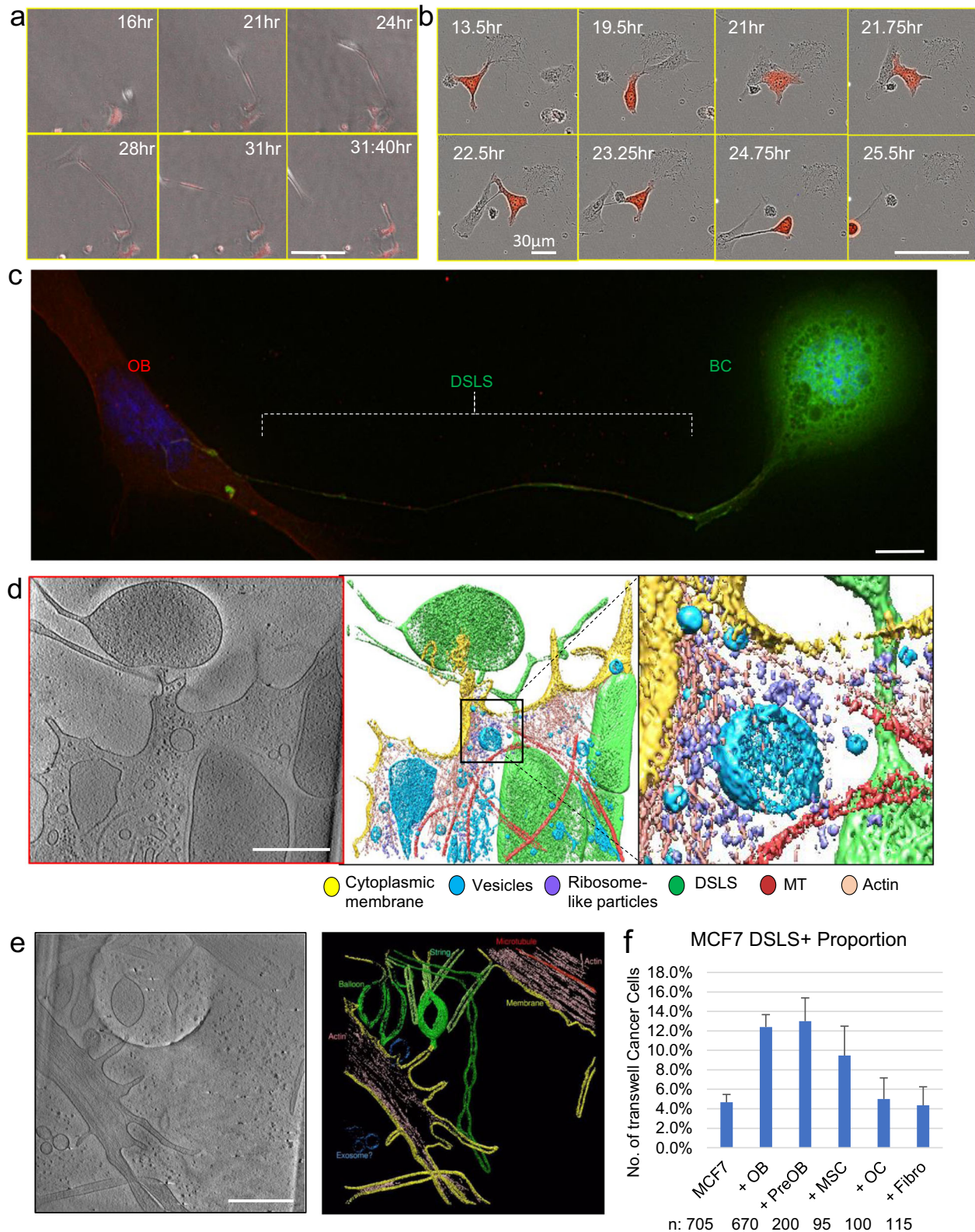
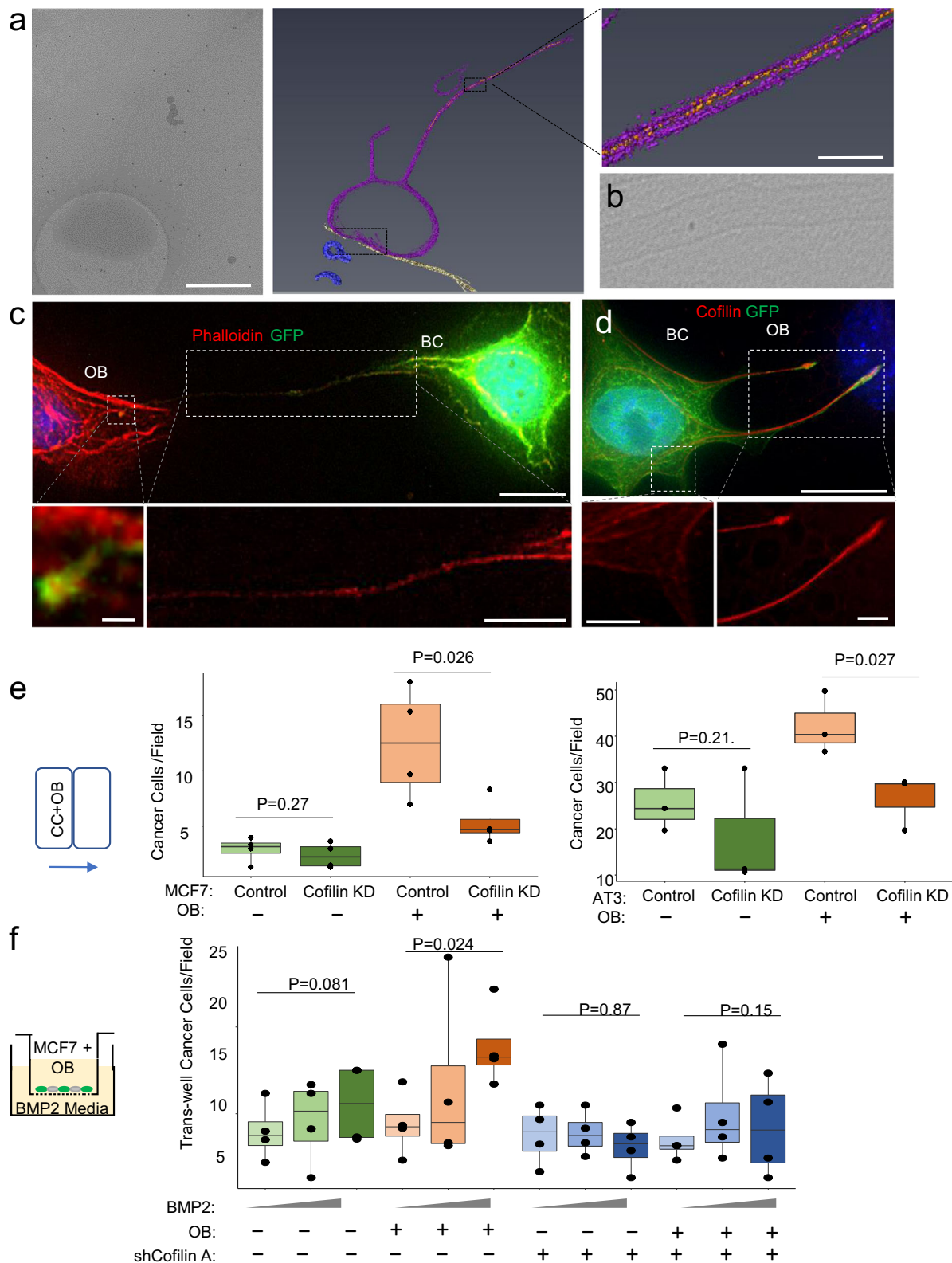


Fig. 2 Luminal breast cancer cells form dendritic spine like protrusions that tether them to mobile osteoblasts. **a** Time-lapse on Cytation of mobile osteoblasts contacting and drawing out cellular protrusion from MCF-7 (red). Scale bar is 100 μ m. **b** Time-lapse on Incucyte S3 of osteoblast (unlabeled) 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).

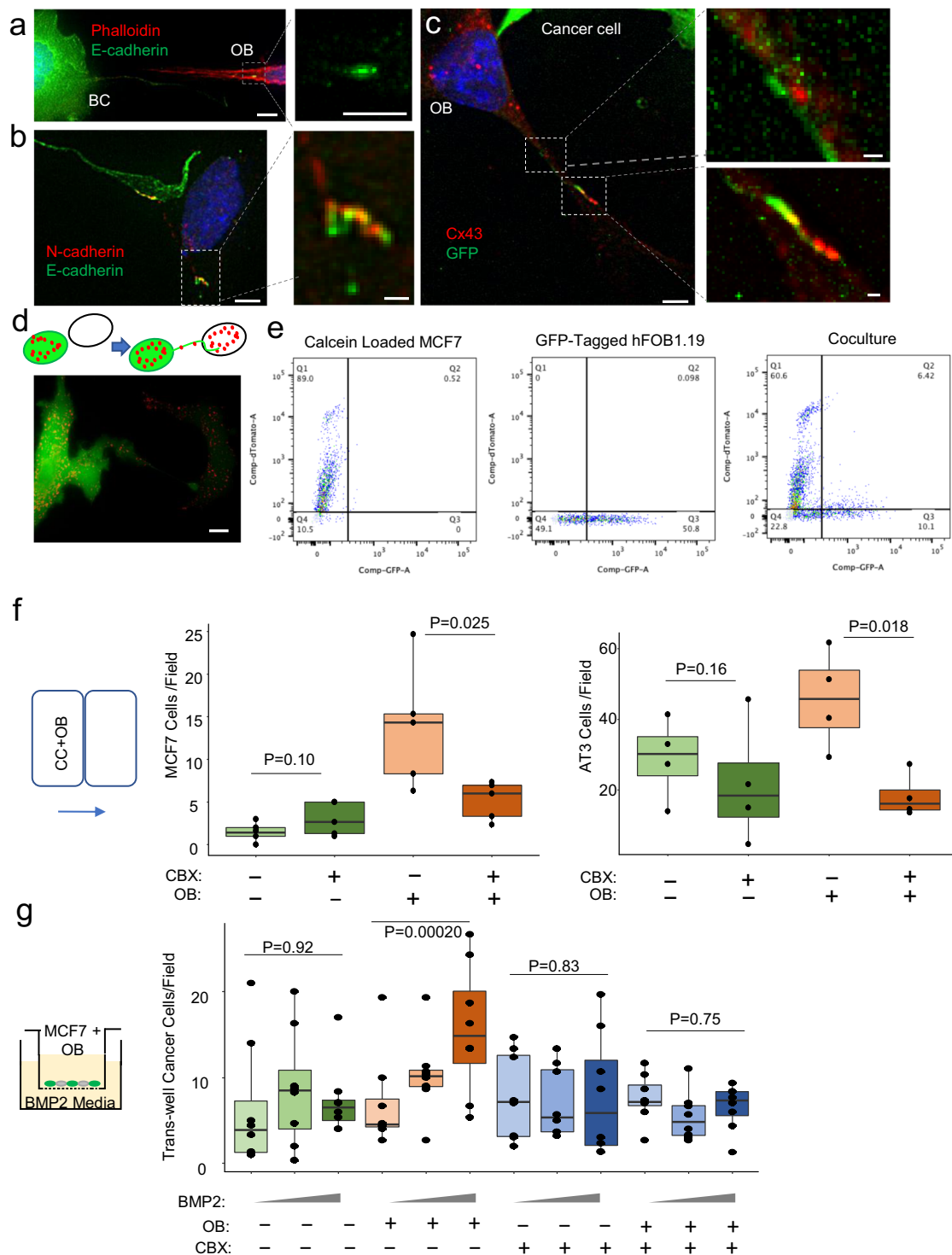
Adherens junctions often precede the development of, and can stabilize GJs, which allow direct substance diffusion between the contacting cells^{31–33}. Connexin 43 (Cx43), a major component of GJ in bone micrometastases¹³, was expressed at the interface between DSLS and osteogenic cells (Fig. 4c). Pre-incubation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

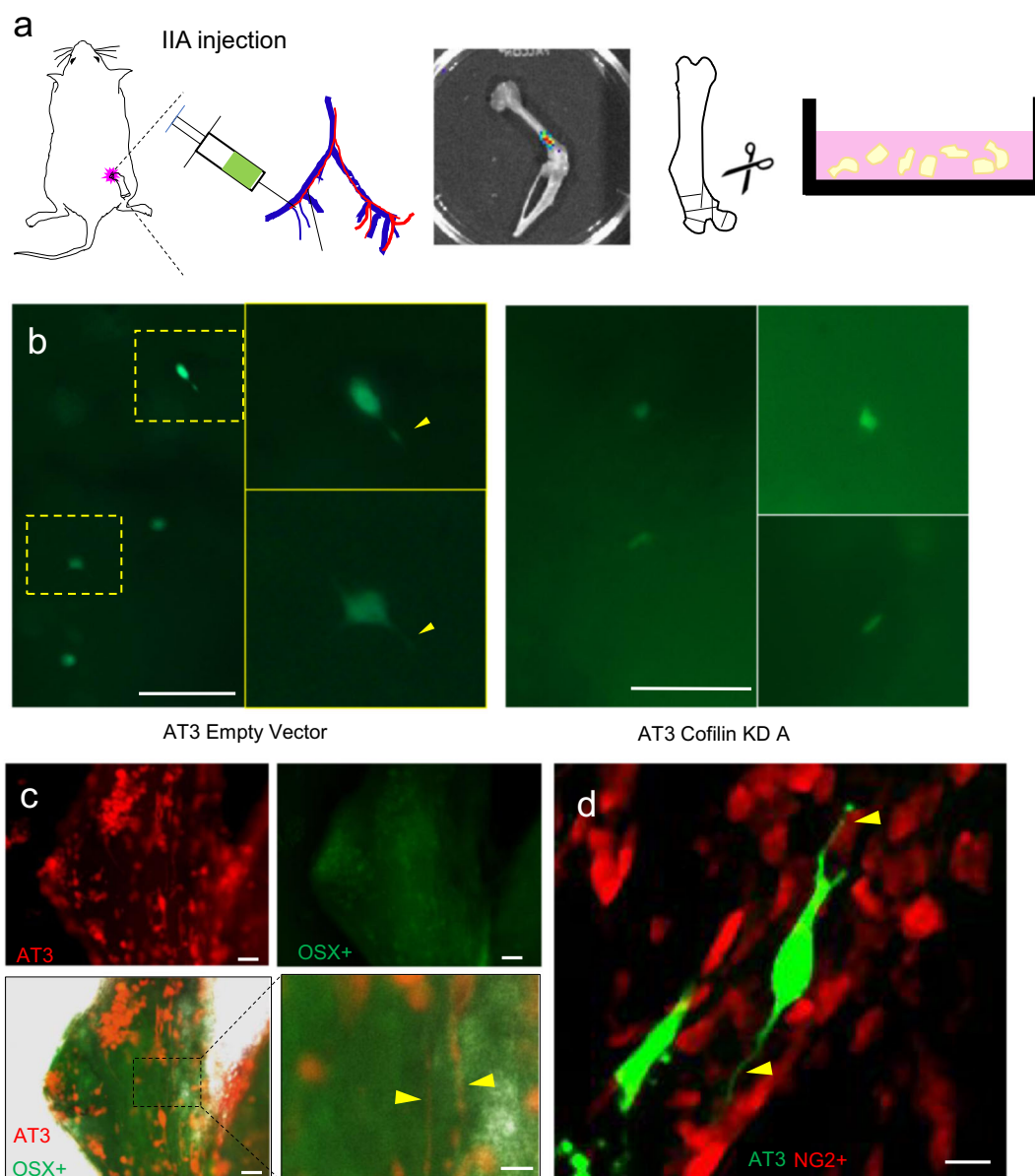


Fig. 5 Evidence of DSLS in breast cancer bone metastasis. Labeled AT3 cells were injected via intra-iliac artery (IIA) injection into C57B/6 mice. Mice were immediately sacrificed, hind limb bones harvested, and cut into small (1–2 mm diameter) pieces. Bone fragments are cultured over the next 3 days 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 × 4k CCD camera and a Gatan Model 626 side-entry cryo-specimen holder (Gatan, Inc.). The nominal magnification used for imaging was ×15,000 with a calibrated pixel size of 7.64 Å/pixel. Data collection was performed using SerialEM⁴⁸ with a target defocus of –6 μ m, a tilt range of –60° to +60° 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.

ADDITIONAL INFORMATION

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Correspondence and requests for materials should be addressed to X.-F.Z.

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