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

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1. Introduction

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

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

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

2. Keywords:

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

3. Accomplishment

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

Subtask 1: Tumor burden measurement (Month 1-24). We expect to use five PDXs (2 ER+, 1 Her2+ and 2 triple negative) and five cell lines (the same subtype distribution). The total # of models will be 10. Each model will need 55 mice. PDXs will be transplanted into SCID/Beige mice and cell lines will be transplanted into Athymic nu/nu mice. These mice will be divided into treated and untreated. Treatments: tamoxifen, fulvestrant, ovariectomy, and lapatinib. Measurement: Weekly bioluminescence imaging and tumor volume measurement. Some mice will be euthanized at intermediate time points for Subtasks 2 and 3 below.

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

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

This subtask was deprioritized due to our discovery of a more interesting phenomenon related gap junctions formed between cancer cells and osteoblasts, which was published on *Cancer Cell*[1], as reported last year. Afterwards, we returned to this subtask and examined the cell-biological features of bone micrometastases with a focus on ER+ breast cancers, as this subtype exhibits stronger bone tropism in the clinic. Instead of inspecting proliferation, survival, and self-renewal individually, we took advantage of recent technical advancement in the lab and performed unbiased profiling of cancer cells extracted from the bone microenvironment.

We used multiple approaches including 1) translating ribosome affinity purification (TRAP) followed by RNA-seq (TRAP-seq) to profile transcriptome in cancer cells that are interacting with osteogenic cells in 3D suspension co-cultures without dissociating the two cell types, and 2) reverse phase protein array (RPPA) to profile over 300 key proteins and phosphor-proteins in cancer cells that have been extracted from the bone microenvironment (**Fig. 1A**). In 1), we also applied fulvestrant, tamoxifen and estradiol to the co-cultures to perturb ER signaling. In 2), we included SCP2, a genetically homogenous population that exhibits enhanced ability of bone colonization. According to TRAP-seq, over 1,100 genes are significantly increased by MSC co-cultures (FDR < 0.05 and fold change > 2), which is a large number and indicates a global phenotypic alteration. Indeed, using PAM50 signatures, we observed a dramatic shift from luminal to basal subtype **(Fig. 1B)**. Consistently, examination of the 50 HALLMARK pathways in MSigDB uncovered several significant changes including the decrease of ER signaling and increase of EMT (**Fig. 1B**), both of which indicated dedifferentiation and stem-like activities. In addition, we also identified GJA1, which encode Connexin 43, the gap junction component playing important roles in cancer-osteogenic interaction[1], is induced by co-culture of MSCs (**Fig. 1B**), suggesting a potential link to calcium signaling that is activated by calcium flux from MSCs to cancer cells[1]. 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 activity), stemness-related pathways (e.g., WNT and Notch signaling), and receptor tyrosine kinase signaling (**Fig. 1C**). These findings indicate that the osteogenic microenvironment induces an epigenomic landscape alteration in ER+ breast cancer cells toward more ER-independent and stem-like states.

Using RPPA, we compared the original MCF-7 cells and SCP2 with their derivatives that were extracted from bone lesions, which we named "bone-entrained" cells. The bone-entrained cells exhibited reduced ER signaling (**Fig. 1D**), enhanced stemness (**Fig. 1D**), increased mesenchymal properties (**Fig. 1E**), and strikingly, increased RTK expression (**Fig. 1F**). Notably, it should be stressed that these changes are persistent even after cancer cells are extracted from the bone microenvironment, and represent some "epigenetic memory". In fact, one of the phenotypic consequences, the endocrine resistance, also persists in vitro for at least 8 passages after extraction of cancer cells from the bone (**Fig. 1G**).

Taken together, we have exceeded the research goals original proposed in our project and observed a phenotypic landscape shift of ER+ breast cancers in the bone microenvironment. We are in the process of repeating these analyses on ER- breast cancers, which will be reported after next grant year.

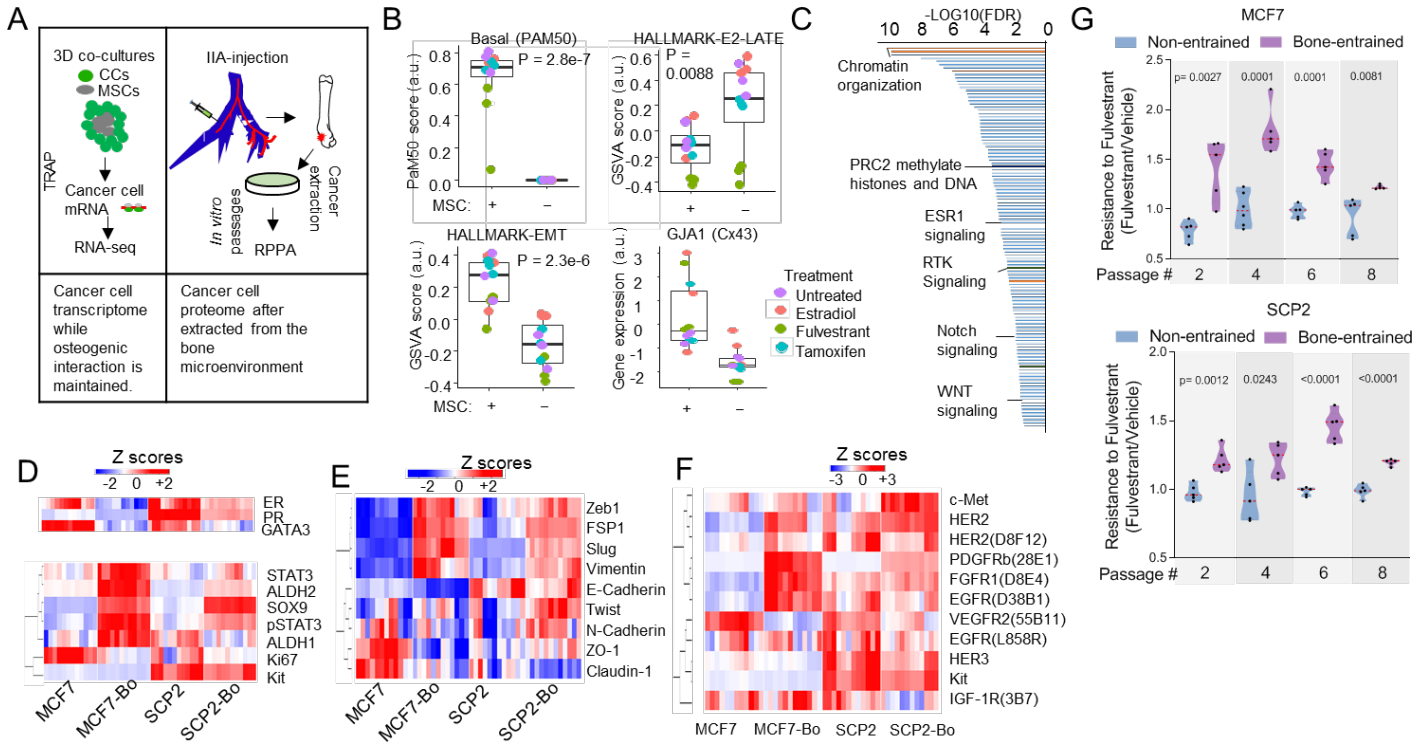

Figure 1. Global epigenomic reprogramming effects of the osteogenic cells on ER+ breast cancer cells (A) Schematics show experimental strategies to profile transcriptome of cancer cells while interacting with MSCs via TRAP-seq of 3D co-cultures of MCF-7 cells and MSCs (left) and partial proteome of MCF-7 parental or SCP2 cells extracted from IIA-derived bone lesions and expanded in vitro by several passages (right). (**B**) Box plots show metrics of indicated pathways and expression of single gene GJA1 in MCF-7 cells with or without 3D co-cultures of MSCs using TRAP-seq data. Various endocrine treatments were applied which are shown by different colors. (**C**) Output of gene ontology enrichment analysis using differentially expressed genes between MCF-7 cells with and without co-cultures of MSCs in 3D. Pathways that attract attention are indicated. (**D-F**) Selected groups of (phosphor)-proteins in parental MCF-7, bone-entrained MCF-7 (MCF7-Bo), original SCP2, and bone-entrained SCP2 (SCP2-Bo) assessed by RPPA. (**G**) Violin plots show responses of MCF7-Bo and SCP2-Bo cells to fulvestrant as a function of cell passages in vitro. P values are computed based on Student's t tests.

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

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

As mentioned in last year's report, this work has been finished and the resultant mouse colonies are being expanded for ongoing experiments. The conditional knockout of gap junction has been utilized in our published work to demonstrate its relevance in bone colonization[2]. The conditional knockout of N-cadherin also hindered bone metastasis as we published[3]. Beyond these observations, we also noticed interesting effects of conditional knockout of N-cadherin on a phenomenon we named "migration-by-tethering", which will be elaborated below.

Subtask 2: Repeat experiments in Major Task 1 in the conditional N-cadherin KO models, and Test if the abolishment of cancer-niche interaction in conditional N-cadherin KO mice reverses the therapeutic responses of BMM. TetO-Osx-cre-GFP; Cdh2$^{f/f}$ mice and TetO-Osx-cre-GFP; Cdh2f/f; Rag1-/- mice and their littermate female mice lacking Osx-Cre will be subjected to experiments as in Task #1. About 700 mice will be bred at this stage. Except that a few male mice carrying the wanted phenotype will be kept for strain maintaining, most male mice (estimated to be 340) will be euthanized right after genotyping. (Month 24-36)

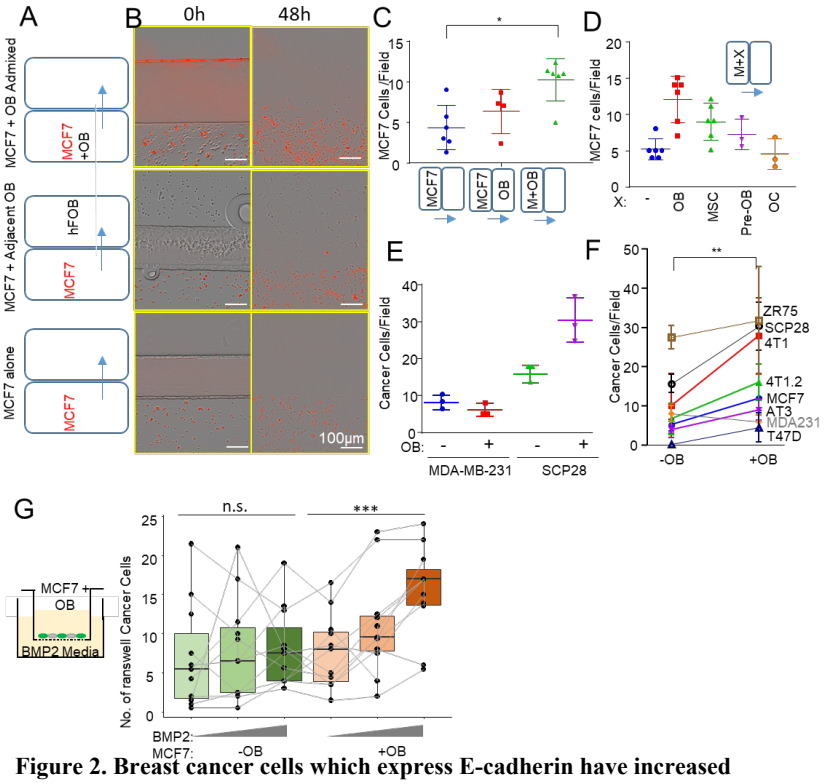

Figure 2. Breast cancer cells which express E-cadherin have increased migration when cultured with osteogenic cells *in vitro*. (A). Ibidi silicone removable chambers were used to quantify migration by breast cancer cells in monoculture, in co-culture with osteoblasts in adjacent chambers, or in direct 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. **(C).** Dot plot showing the number of cells escaped from their original enclosure per field for MCF7 alone, in co-culture with OBs in adjacent regions, or in co-culture with osteoblasts in the same region after 48 hours. Each dot represents a biological replicate. **(D).** Dot plot shows the number of cells escaped per field for MCF7 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 286.3 cells) after 48 hours. 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 hours. Each dot represents a technical replicate. **(F).** Dot plot shows the combined data for cells escaped per field for various E-cadherin expressing breast cancer cell lines alone and with osteoblasts. For contrast MDA-MB-231, which lacks E-cadherin expression, is also shown (not included in statistical comparison). **P<.01 students paired t-test. **(G).** Box and dot plots shows the trans-well migration (a diagram shown in the left) of MCF7 and AT3 cells toward a gradient (0, 1, and 5 μM) of Bone Morphogenetic Protein-2 (BMP-2) with or without admixture of OBs. Each dot represents a biological replicate generated from an independent experiment. The dots generated by the same experiment under different conditions are connected by grey lines. *P<.05; **P<.01; ***P < 0.001, as determined by analysis of covariance (ANCOVA).

In last year's report, we stated that the usage of N-cadherin KO mice was deprioritized because of a transient shift of focus to gap junctions. After the gap junction story was published, we returned to in-depth investigation on N-cadherin. Serendipitously, we uncovered a potentially very novel role of N-cadherin in bone colonization.

Our previous studies supported by this grant have established a central role of osteogenic cells in early-stage bone colonization. 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, 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. 2A**. 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 works[4].

Inconsistent to our hypothesis, MCF-7 cells did not exhibit chemotactic movement toward osteogenic cells in the adjacent chamber (**Fig. 2B and 2C**). 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. 2B**). 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. 2B,C**). Real-time imaging revealed that cancer cells stick to the much more mobile osteogenic cells and be "dragged" to move, 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 RAW286.7 osteoclasts failed to increase of MCF-7 motility (**Fig. 2D**). Thus, it appeared that MBT is specifically 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. All of

these lines exhibited similar MBT properties (**Fig. 2E,F**). In particular, SCP28 is a single cell-derived, bone-seeking subpopulation of MDA-MB-231. One difference between SCP28 and the parental population is the expression of E-cadherin. Interestingly, migration of MDA-MB-231 parental cells did not increase when co-cultured with osteogenic cells (**Fig. 2E**), 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. 2F**).

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 trans-well assays in which cancer cells and/or osteogenic cells are admixed in the upper chamber and BMP2-containing media is placed in the bottom chamber. As expected, trans-well migration of MCF-7 cells did not change by BMP2 when they are cultured alone, but significantly increased when osteogenic cells are co-cultured (**Fig. 2G**) – supporting that osteogenic cells and MBT may drive relocation of cancer cells toward sites releasing osteogenic signals.

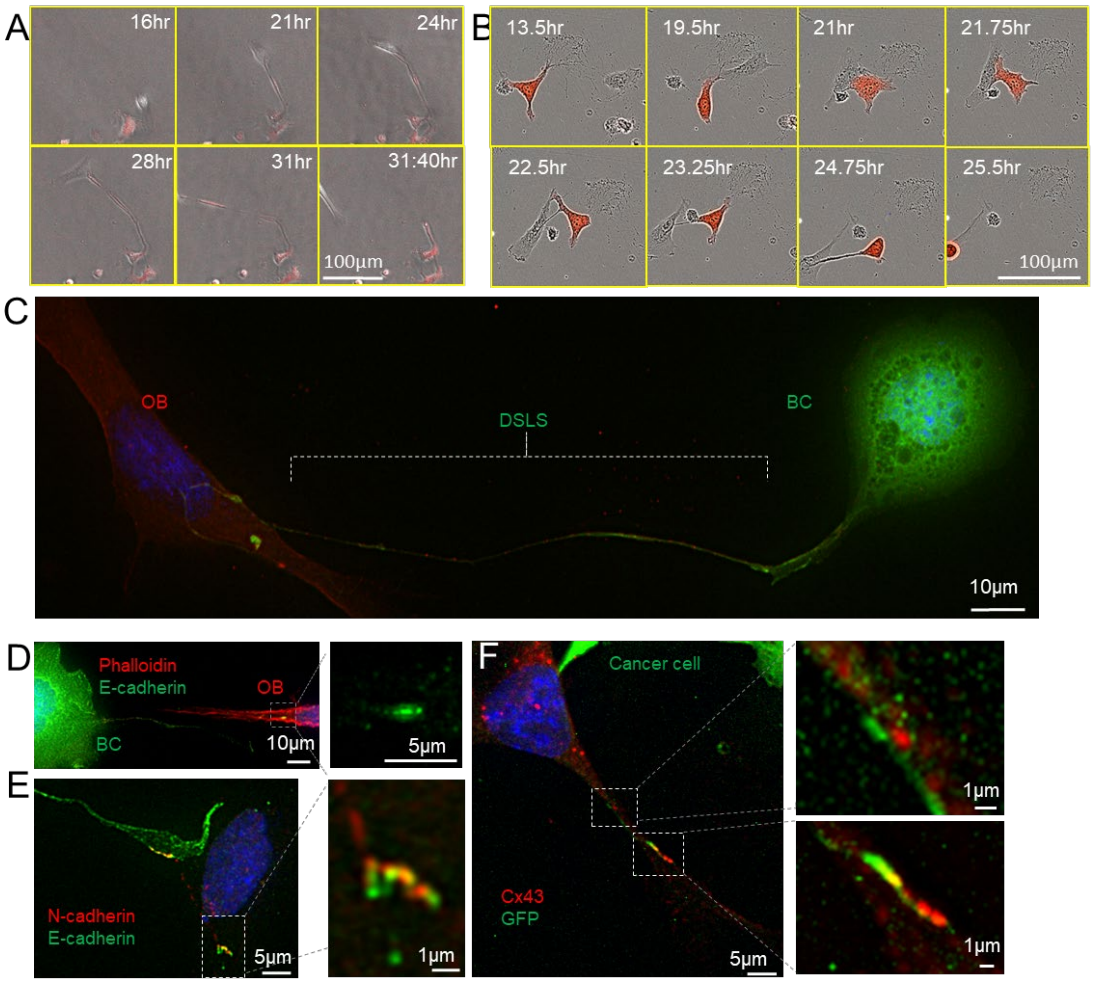

Figure 3. Luminal breast cancer cells form dendritic spine like protrusions (DSLS) that tether them to mobile osteoblasts (A). Time-lapse on Cytation of mobile osteoblasts contacting and drawing out cellular protrusion from MCF7 (red). **(B).** Timelapse on Incucyte S3 of osteoblast (unlabeled) drawing out cellular tether protrusion that leads to MCF7 (RFP-labeled,) migration. **(C)**. Deconvolution imaging of MCF7 cell (green) with dendritic spine-like structures (DSLS) protrusions attached to OBs (red). **(D)**. MCF7 E-cadherin (green) expressed where DSLS contacts osteoblast, here visible with phalloidin staining (red). **(E)**. MCF7 E-cadherin (green) binding osteoblast N-cadherin (red) where DSLS contacts osteoblast. **(F)**. Osteoblast gap junction protein connexin 43 (Cx43 – red) at the site of MCF7 (green) DSLS contact.

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. 3A**). In some cases, the connection eventually led to co-migration of cancer cells in an abrupt manner (**Fig. 3B**). Through deconvolution microscopy, we observed that the protrusion structure morphologically resembles dendritic spines of neurons – 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. 3C**). Sometimes additional spherical structures can be found in the middle of the neck. Therefore, we named this protrusion dendritic spine-like

structure, or DSLS. Very interestingly, subsequent immunofluorescence staining revealed that E-cadherin is often concentrated at the head of DSLS (**Fig. 3D**), and co-localizes with N-cadherin (**Fig. 3E**). Moreover, gap junction was also observed between the tip of DSLS and osteogenic cells (**Fig. 3F**).

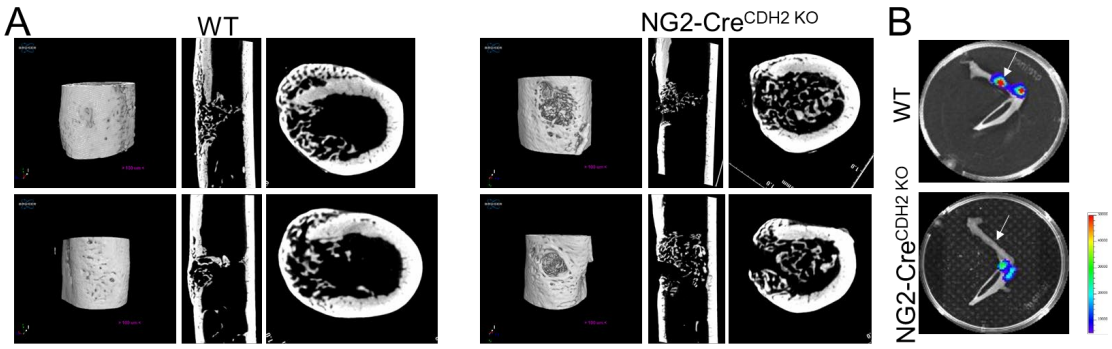

Figure 4. Conditional knockout of N-cadherin (CDH2) in osteogenic cells (NG2+ mesenchymal stem cells and their descendent cells) slowed down bone healing and blocked bone colonization of DTCs to the injured sites. (A). Micro-CT images show the healing of drilled bones in mice that are wild type (WT) or conditional KO of N-cadherin. **(B).** Bioluminescence imaging of bone colonization of DTCs to the drilled injure sites (white arrows).

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 injured site and stimulate subsequent osteogenic differentiation. Very importantly, N-cadherin may mediate the physical contact between cancer cells and MSCs during MBT, and thereby representing a key target that can be exploited to disrupt MBT. As a proof-of-principle, we tested how DTCs may translocalize to injured sites (by drilling) in mice with conditional knockout of N-cadherin. Preliminary data clearly indicated that lack of N-cadherin slow down bone healing (**Fig. 4A**), and at the same time, abolished colonization to injured sites of bone (**Fig. 4B**). Going forward, we will continue to expand this experiment and explore the ability to prevent overt bone metastases by targeting N-cadherin and breaking MBT.

Major Task 3: To establish and validate BICA

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

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

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

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

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

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

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

PDXs will be transplanted into SCID/Beige mice and cell lines will be transplanted into Athymic nu/nu mice. (Month 30-48)

As reported in last year's update, we have made significant findings in fulfilling this research subtask. Briefly, agents targeting gap junctions have been discovered by BICA and validated in vivo. In the current report period, we continued this line of research and extended BICA test to a number of our therapeutic agents. The focus was on pathways that render DTCs and BMMs resistant to endocrine therapies, including calcium signaling and FGFR signaling in multiple models (**Fig. 5**). The rationale behind testing this drug was provided in **Fig. 1** and associated description.

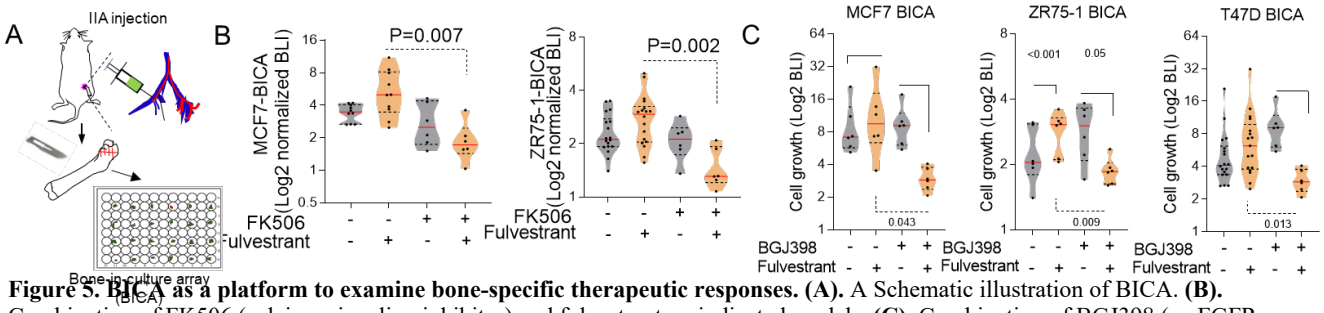

Figure 5. BICA as a platform to examine bone-specific therapeutic responses. (A). A Schematic illustration of BICA. **(B).** Combination of FK506 (calcium signaling inhibitor) and fulvestrant on indicated models. **(C).** Combination of BGJ398 (an FGFR inhibitor) and fulvestrant on indicated models. P values are based on Student's T tests.

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

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

This subtask has been accomplished, and several new findings have been made as reported before. We are extending bioinformatics analyses to pathways that mediate phenotypic plasticity and endocrine resistance in the bone microenvironment. Some of the results are shown in **Fig. 1**.

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

This effort has started and generated promising outcome. Representative results are shown in **Fig. 3**. We will systematically report all relevant results upon the completion of the 5th grant year.

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

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

As reported before, we have finished screening of two small libraries: one contains FDA-approved anti-neoplasm drugs, and the other contains compounds targeting epigenomic modulators[2,3]. Because of the interesting discoveries in the first two screening, we have decided to deprioritize screening of the 3rd and 4th libraries. We propose to conduct more screening later in the project should the need arises for more candidate drugs.

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

This subtask has been accomplished and reported last year.

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

Toward this end, we have started collaboration with Dr. Han Xiao's laboratory at Rice University to conjugate chemical drugs with therapeutic antibodies to achieve better efficacies and specificities against DTCs and BMMs. Dr. Xiao pioneered a technology named pClick. pClick is a radically new antibody conjugation

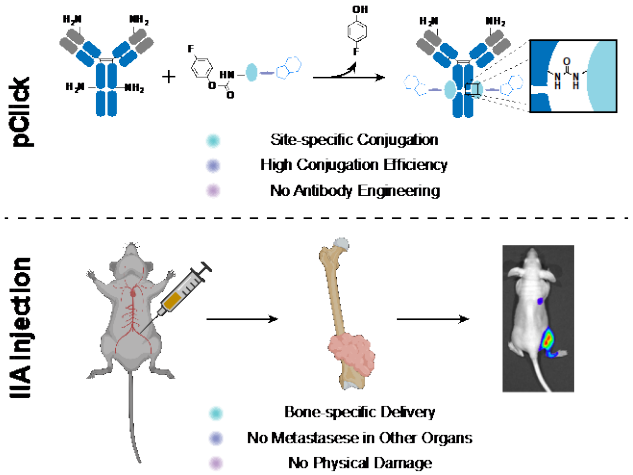

Figure 6. Scheme of proximity-induced antibody conjugation technology (pClick) and intra-iliac artery (IIA) injection.

technology for precision labeling of native antibodies with payloads containing different chemical, physical, or biological properties under mild conditions. This technology does not rely on antibody engineering or additional UV/chemical/enzymatic treatment, thus it is applicable to most reported therapeutic antibodies. In combination with our in vivo bone metastasis models, we will determine if we can increase the specificity and efficacies of drugs identified in our previous aims (**Fig. 6**).

As a starting point, we conjugated a bisphosphonate that is known to have bone-specific effects, alendronate (ALN), to therapeutic antibodies with established targets and efficacies, trastusumab (Tras). The "pClicked" new drug, namely ALN-conjugated Tras exhibited superior effects on Her2+ breast cancer cells in the bone compared to ALN or Tras alone (**Fig. 7A**). Strikingly, these is even an effect of ALN-conjugated Tras on non Her2+ cancer cells such as MCF-7 (**Fig. 7B**). Taken together, these data support pClick as a promising strategy to optimize and modify compounds that were identified in previous tasks. Going forward, we will consider using pClick to conjugate the following drugs to specific antibodies, as will be elaborated below.

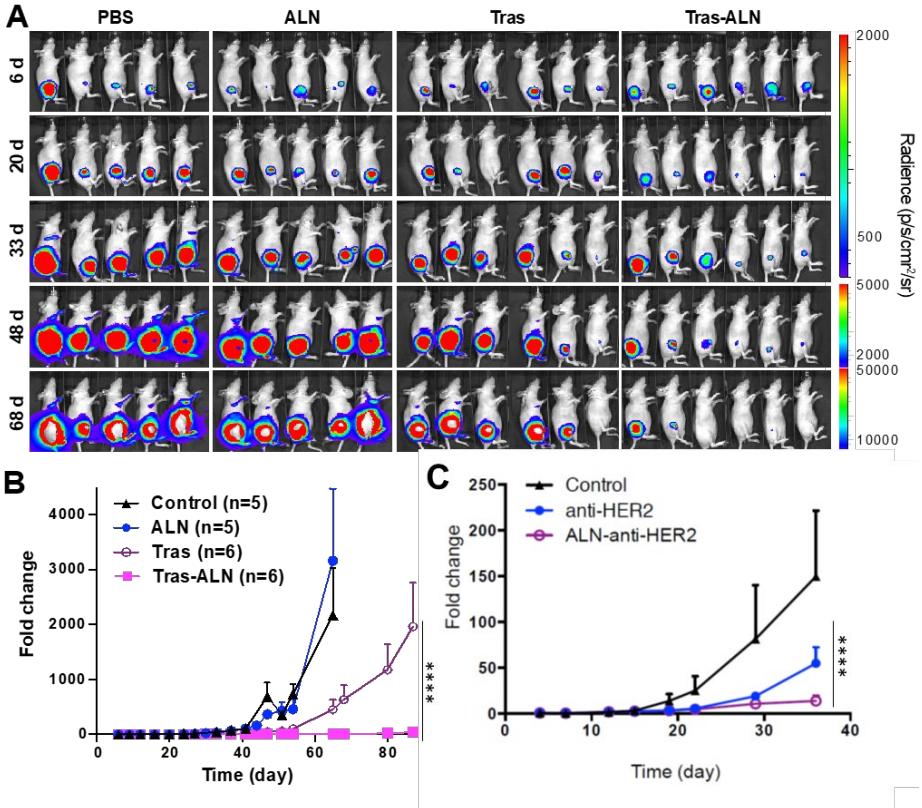

Figure 7. In vivo comparison of Tras and Tras-ALN (A) Tumor burden of MDA-MB-368 (ER+/Her2+) bone metastases was monitored by weekly bioluminescence imaging, and (B) quantified by the radiance detected in the region of interest. (C) Tumor burden of MCF-7 (ER+/Her2-) bone metasasis burden $_{****}P < 0.0001$ and n.s. = $P > 0.05$.

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

So far, our studies based on BICA, in vivo experiments, and bioinformatics analyses have identified a number of pathways that may play important roles in bone colonization (e.g., pathways examined in **Fig. 1**) and that may be drug targets for future clinical studies. Of these, EZH2 is of pivotal interest (as indicated by **Fig. 1C**). EZH2-mediated epigenomic reprogramming is a leading candidate because it is known to globally regulate cancer stemness[5,6] and therapeutic agents are already developed and being used in clinical investigations (albeit for other cancers)[7]. Our Going forward, we will dissect the molecular mechanisms through which EZH2 is activated by the bone microenvironment and subsequently enhance stem cell properties. We will also test if a transient but efficient blockade of EZH2 may impede metastasis seeding from bone lesions.

Our preliminary studies strongly indicate EZH2 activation specifically in cancer cells colonizing bone (**Fig. 8A,B**). Apparently, interaction with MSCs is sufficient to enhance EZH2 (**Fig. 8C,D**). Our previous studieshave

demonstrated that cancer cells and osteogenic cells including MSCs can establish heterotypic adherens and gap junctions (hAJs and hGJs), through which the mTOR and calcium signaling can be activated in cancer cells[1,8]. We will examine if these pathways can in turn enhance EZH2 expression. In addition, it is known that the FGFR pathway increases EZH2 expression[9]. Interestingly, both FGF ligands and receptors are increased in bone metastases compared to matched primary tumors[10]. Therefore, we will also test the role of FGFR pathway in regulating EZH2 expression in the bone microenvironment.

We will interrogate the mTOR, calcium signaling, gap junctions, and FGFR pathway in 3D cancer-MSC co-cultures as well as in bones. Preliminary studies already showed that treatment of CaCl2 or recombinant FGF2 increased EZH2 expression at RNA and protein levels, respectively (**Fig. 8E,F**).

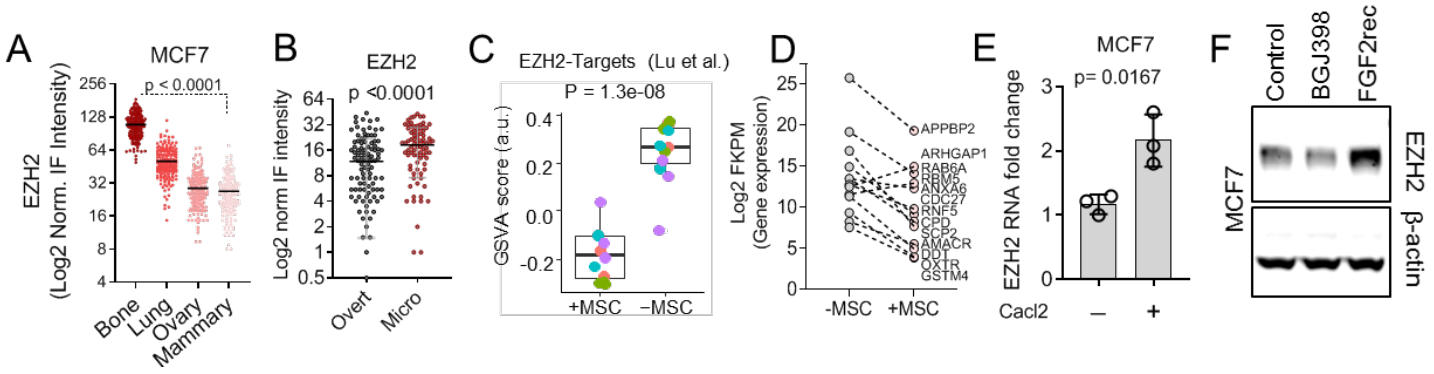

Figure 8. Preliminary evidence supporting the enhanced EZH2 activates in the bone lesions, as a consequence of calcium and FGF signaling. **(A)** Immunofluorescence (IF) intensity of EZH2 in MCF7 cells colonizing bone and other organs. P values are computed based on one way ANOVA. **(B)** The EZH2 signal appeared to be even stronger in microscopic bone lesions (Micro) as compared to overt bone lesions (defined by involvement of osteoclasts). **(C)** TRAP-seq revealed that EZH2 targets are silenced (higher EZH2 activities) when cancer cells are co-cultured with MSCs. **(D)** Examples of EZH2 target gene expression with or without MSC co-culturing. **(E)** Ca2+ treatment increase RNA level of EZH2. **(F)** Treatment of recombinant FGF2, the FGF most abundant in the bone microneviornment, enhance EZH2 protein level.

4. Impact

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

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

5. Changes/Problems

So far, we have been making rapid progress and made multiple discoveries. Based on these discoveries, we have slightly adjusted our future plan. Instead of doing more exploratory studies (e.g., drug screening), we are deepening our studies on intriguing pathways and corresponding inhibitors that were already discovered. Specifically, we will focus on 1) how targeting EZH2 may revert bone microenvironment-induced endocrine resistance, 2) pilot studies using pClick technology to optimize existing drugs for DTC/BMM treatment, and 3)

how gap junctions, calcium signaling and the ER pathways interact with one another. We will proactively seek opportunities and additional funding to translate our current findings.

6. Products

Publications:

1. Wang H, Zhang W, Bado I, Xiang H.-F. Zhang. (2019) "Bone Tropism in Cancer Metastases." Cold Spring Harb Perspect Med. 2019 Oct 24. PMID: 31615871 DOI: 10.1101/cshperspect.a036848
2. Bado I, **Xiang H.-F. Zhang**. (2020) "Senesce to Survive: YAP-Mediated Dormancy Escapes EGFR/MEK Inhibition." Cancer Cell, 13;37(1):1-2. doi: 10.1016/j.ccell.2019.12.008.
3. Gao Y, Bado I, Wang H, Zhang W, Rosen JM, and **Xiang H.-F. Zhang**. (2019). "Metastasis Organotropism: Redefining the Congenial Soil." Dev Cell, 2019, 49(3):375-391. PMID: 31063756 PMCID: PMC6506189
4. Zhang W, Lo HC, Bado I, Wang H, and **Xiang H.-F. Zhang**. (2019) *Bone metastasis: find your niche and fit in*. Trends in Cancer. 5(2):95-110.

Grants (pending):

1. NCI R01 CA183878 (Role: PI, renewed): "Osteoclast-independent mechanisms underlying early-stage bone colonization".
2. NCI R01 CA251950 (Role: PI, scored 6%): "Mechanistic and therapeutic investigation of secondary metastatic seeding from breast cancer bone lesions".
3. DoD CDMRP BC191140 (Role: PI, recommended for funding, Expansion Award of this project): "The epigenetic adaptation of ER+ breast cancer cells to the bone microenvironment".
4. Laura Ziskin Award (Role: PI): "Resistance mechanisms of immune checkpoint blockade therapies in TNBC".

7. Participants & Other Collaborating Organizations

Name:	Chenghang Zong
Project Role:	Co-investigator
Researcher Identifier:	N/A
Nearest person month worked	0.36
Contribution	Dr. Zong is an expert of single-cell sequencing, and is helping us establish protocols to sequence BMM transcriptomes, which is critical to delineate molecular mechanisms underlying endocrine resistance of BMM.
Funding Support	Dr. Zong was also supported by NIH New Innovator1DP2EB020399-01
Name:	Yongcheng Song
Project Role:	Co-investigator
Researcher Identifier:	N/A
Nearest person month worked	0.6
Contribution	Dr. Song is an expert of chemical synthesis and modification of

	drugs. He is helping us to improve bioavailability and pharmacokinetics of potential bone metastasis drugs.
Funding Support	Dr. Song was also supported by NIH R01NS080963, Cancer Prevention and Research Institute of Texas RP140469 and RP150129
Name:	Michael Lewis
Project Role:	Co-investigator
Researcher Identifier:	N/A
Nearest person month worked	0.6
Contribution	Dr. Lewis established a cohort of PDX models. He is helping us utilize PDX models to generate metastasis models for mechanistic and therapeutic studies.
Funding Support	Dr. Lewis is also supported by fundings from NSF (1263742), NIH (CA179720) and Helis Foundation.
Name:	Hai Wang
Project Role:	Instructor
Researcher Identifier:	N/A
Nearest person month worked	12
Contribution	Dr. Wang specialize in bone metastasis research techniques and is leading the efforts of establishing BICA.
Name:	Yang Gao
Project Role:	Postdoctoral Fellow
Researcher Identifier:	N/A
Nearest person month worked	6
Contribution	Dr. Yang focuses on the role of estrogen receptors in driving bone microenvironment-dependent endocrine resistance.
Name:	Zhan Xu
Project Role:	Postdoctoral Fellow
Researcher Identifier:	N/A
Nearest person month worked	12

Contribution	Dr. Xu focuses on the roles of various bone microenvironment niches during bone metastasis colonization.
Name:	Emmale Davis
Project Role:	Research Technician
Research Identifier:	N/A
Nearest person month worked	4
Contribution	Assistance to animal experiments.

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

Developmental Cell
Review

Metastasis Organotropism: Redefining the Congenial Soil

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Metastasis is the most devastating stage of cancer progression and causes the majority of cancer-related deaths. Clinical observations suggest that most cancers metastasize to specific organs, a process known as "organotropism." Elucidating the underlying mechanisms may help identify targets and treatment strategies to benefit patients. This review summarizes recent findings on tumor-intrinsic properties and their interaction with unique features of host organs, which together determine organ-specific metastatic behaviors. Emerging insights related to the roles of metabolic changes, the immune landscapes of target organs, and variation in epithelial-mesenchymal transitions open avenues for future studies of metastasis organotropism.

Introduction

Metastasis is a process by which cancer cells are dispersed from their primary site of tumorigenesis and disseminated to a different part of the body. It remains the major cause of morbidity and mortality in cancer patients (Chaffer and Weinberg, 2011). Before colonization at secondary sites, cancer cells generally undergo a complicated cascade, including invasion to surrounding tissue, intravasation to the blood vessels, survival in the circulation, and extravasation to colonize and thrive at distant sites (Obenauf and Massagué, 2015).

Metastasis follows a non-random distribution among distant organs, known as "organotropism" or "organ-specific metastasis." Different cancer types and subtypes display distinct organotropisms. For example, prostate cancer preferably relapses in bone while uveal melanoma typically colonizes in liver (Nguyen et al., 2009). Breast cancer can metastasize to different sites, including bone, lung, liver, and brain. However, the luminal subtype has a higher propensity to metastasize to the bone, whereas metastases of triple-negative breast cancer (TNBC) prefers visceral organs (Chen et al., 2018; Wu et al., 2017). Accumulating evidence suggests that organotropism is regulated by multiple factors, including the circulation pattern, tumor-intrinsic factors, organ-specific niches, and the interaction between tumor cells and the host microenvironment (ME). In this review, we summarize recently emerging concepts and mechanisms underlying organ-specific metastasis.

General Metastasis Mechanisms

Metastatic tumors largely rely on the same driver mutations found in primary tumors (Zehir et al., 2017), suggesting that the hallmark functions for tumor maintenance and progression remain critical in metastases. Regardless of their final destination, the early steps in the cancer metastasis cascade are generally similar among different cancers. For example, the epithelial-mesenchymal transition (EMT) program is thought to play a central role in the departure of cancer cells from primary tumors (Lambert et al., 2017). EMT refers to the loss of epithelial features and acquisition of mesenchymal properties, which is of paramount importance for preparing carcinoma cells to invade the surrounding parenchyma and intravasate to enter the bloodstream. Many EMT-inducing transcription factors (EMT-TFs), including Snail, Slug, Twist, and Zeb1 coordinately regulate this critical process. EMT has been shown to participate in almost all the aspects of tumor dissemination, although some recent studies revealed more complicated dynamics between EMT and metastasis (reviewed in Lambert et al., 2017; Yeung and Yang, 2017). After departure from the primary tumor, cancer cells traveling in the circulation are designated as circulating tumor cells (CTCs). CTCs can disseminate either as single cells or clusters. In order to survive in the bloodstream, CTCs enlist platelets and leukocytes, particularly neutrophils, to evade immunosurveillance (reviewed in Lambert et al., 2017; Riggi et al., 2018). Upon arrival at secondary sites, cancer cells may remain dormant to facilitate adjustment to the new niche environment. Disseminated cancer cells (DTCs) are thought to retain stem cell properties, which may be required to re-initiate tumor growth in distant organs (reviewed in Lambert et al., 2017; Oskarsson et al., 2014).

Mechanisms Underlying Organ-Tropism of Metastasis

Classic "Seed and Soil" Mechanisms

Stephen Paget stipulated that both cancer cell-intrinsic properties ("seed") and the congenial ME ("soil") are essential for metastasis formation (Paget, 1889). Research in the past few decades has greatly enhanced our understanding of the molecular and cellular nature of both "seed" and "soil". In this section, we will review some mechanisms of organ-specific metastasis focusing on the organ-specific ME.

Bone Tropism

Cancers disseminate to bone with different frequencies (Table 1). Breast and prostate cancers are the principal cancers that metastasize to bone (Budczies et al., 2015; DiSibio and French, 2008). Bone and bone marrow comprise unique cell types including osteoblasts, osteocytes, and osteoclasts. Osteoclasts

Table 1. Incidence of Metastasis to Different Organs at Autopsy (%)

Cancer Organ	Bone	Lung	Liver	Brain	Peritoneum	Reference
Breast	71	71	62	22	NA	(Lee, 1985)
Prostate	90.1	45.7	25	1.6	7	(Bubendorf et al., 2000)
Lung	34	–	21	39	NA	(Riihimäki et al., 2014)
Melanoma	48.6	71.3	58.3	54.6	42.6	(Patel et al., 1978)
Pancreas	25	55	62	NA	NA	(Kamisawa et al., 1995)
Renal	44.5	74	34.5	NA	NA	(Johnsen and Hellsten, 1997)
Thyroid	13	78	20	18	13	(Besic and Gazic, 2013)
Gastric	12	15	48	3	32	(Riihimäki et al., 2016b)
Colon	8	32	70	5	21	(Riihimäki et al., 2016a)
Liver	8	44	–	1	9	(Lee and Geer, 1987)
Ovarian	11.2	33.9	47.9	3	83.6	(Rose et al., 1989)

resorb the bone matrix, while osteoblasts refill osteolytic cavities with new bone deposition to either mature into lining cells or to become embedded in the bone matrix to form osteocytes. DTCs can hijack osteoblasts activity and promote osteoclastogenesis, which leads to increased bone resorption (Thomas et al., 1999). This process releases numerous factors from the bone matrix, including calcium, collagens, glycoproteins, hyaluronans, proteoglycans, growth factors, proteinases, and cytokines (Casimiro et al., 2009), which boost the proliferation of tumor cells, thereby creating a vicious cycle between osteoblasts, osteoclasts, and tumor (Figure 1) (Celià-Terrassa and Kang, 2018; Guise, 2002). Therapeutic interventions targeting the vicious cycle, or more specifically the activation of osteoclasts, exhibited clinical benefit in treating bone metastases. The approved drugs include bisphosphonates (Fulfaro et al., 1998), which induce osteoclast apoptosis, and denosumab (Ford et al., 2013), which is a receptor activator of nuclear factor kappa-B ligand (RANKL) antibody preventing osteoclast maturation. These drugs can significantly strengthen bones and delay tumor progression, although their effects on overall survival remain questionable (Croucher et al., 2016). Therefore, more effective treatments are still urgently needed. In recent years, multiple lines of studies further substantiated this paradigm and also began to reveal early-stage events prior to the onset of the vicious cycle (Eyob et al., 2013; Korpal et al., 2009; Lu et al., 2011; Ross et al., 2017; Sethi et al., 2011; Waning et al., 2015; Zhang et al., 2019; Zheng et al., 2017). These studies provided additional therapeutic targets.

Molecular Pathways Involved in Bone Metastasis. Multiple mechanisms have been proposed in bone metastasis (Table S1) and involve both tumor intrinsic and extrinsic factors. Some mechanisms (e.g., CCXL12/CXCR4-mediated chemotaxis) are common among different cancer types (e.g., Table S1; Taichman et al., 2002; Shiozawa et al., 2011; Zhang et al., 2009, 2013), whereas many other mechanisms appear to be cancer type- or even subtype-specific. The expression of estrogen receptor (ER) defines the largest subtype of breast cancer (ER+), which exhibited a stronger bone-tropism as compared to ER− subtype (Kennecke et al., 2010). ER+ breast cancer appears to adopt different mechanisms for bone colonization (Table S1; ER+ oriented) as compared to ER− (Table S1: TNBC and HER2+ oriented). Androgen receptor (AR) is a major driver of prostate cancer, and it has long been thought that development of castration resistance (i.e., independence of AR signaling) is associated with bone metastasis. However, recent systematic studies identified three clusters of prostate cancer (PCS1, PCS2, and PCS3) with PCS2 displaying higher AR signaling and bone metastasis, indicating an unexpected role of AR in bone metastasis (Thysell et al., 2017; You et al., 2016). Interestingly, a recent study found that TMPRSS2-ERG gene fusions can promote osteoblastic bone metastasis in prostate cancer (Delliaux et al., 2018), indicating that specific mutations drive bone metastasis. In lung cancer, several studies suggest the requirement of epithelial markers such as CD24, discoidin domain receptor-1 (DDR1), and melanoma cell adhesion molecule (MCAM) for bone metastasis (Table S1; lung cancer). Limited studies for other cancers including melanoma, myeloma, and bladder cancer have been reported for bone metastasis (Table S1).

Metastatic Niches in Bone. The most studied bone niches include the hematopoietic stem cell (HSC), osteoblastic, vascular endothelial, and neural niches (Calvi et al., 2003; Ding and Morrison, 2013; Katayama et al., 2006). These niches are essential for normal bone development and maintenance. It is increasingly apparent that various niches sustain different stages of cancer metastasis (Celià-Terrassa and Kang, 2018; Ren et al., 2015). For example, the high vascularization of the bone may contribute to cancer progression and dissemination. Indeed, several studies indicated that the perivascular niche maintains metastatic dormancy through cancer-endothelium interactions (Ghajar et al., 2013; Price et al., 2016). The osteogenic niche, on the other hand, was found to promote metastasis progression (Wang et al., 2015). In prostate cancer, the tumor-promoting function of osteoblasts was associated with the HSC niche, suggesting a direct competition between cancer cells and HSCs (Shiozawa et al., 2011). These findings are intriguing, but much remains to be learned. Both endothelial cells and osteogenic cells are highly heterogeneous in bone and bone marrow (Bussard et al., 2008; Yu and Scadden, 2016) and exhibit interactions that are temporally and spatially dynamic. For instance, the type H endothelium ($CD31^{high}$/$Endomucin^{high}$ sinusoidal vessels enriched in growth plates of long bones) maintains perivascular osteoprogenitors, coupling osteogenesis, and angiogenesis (Kusumbe et al., 2014). A recent study, employing continuous

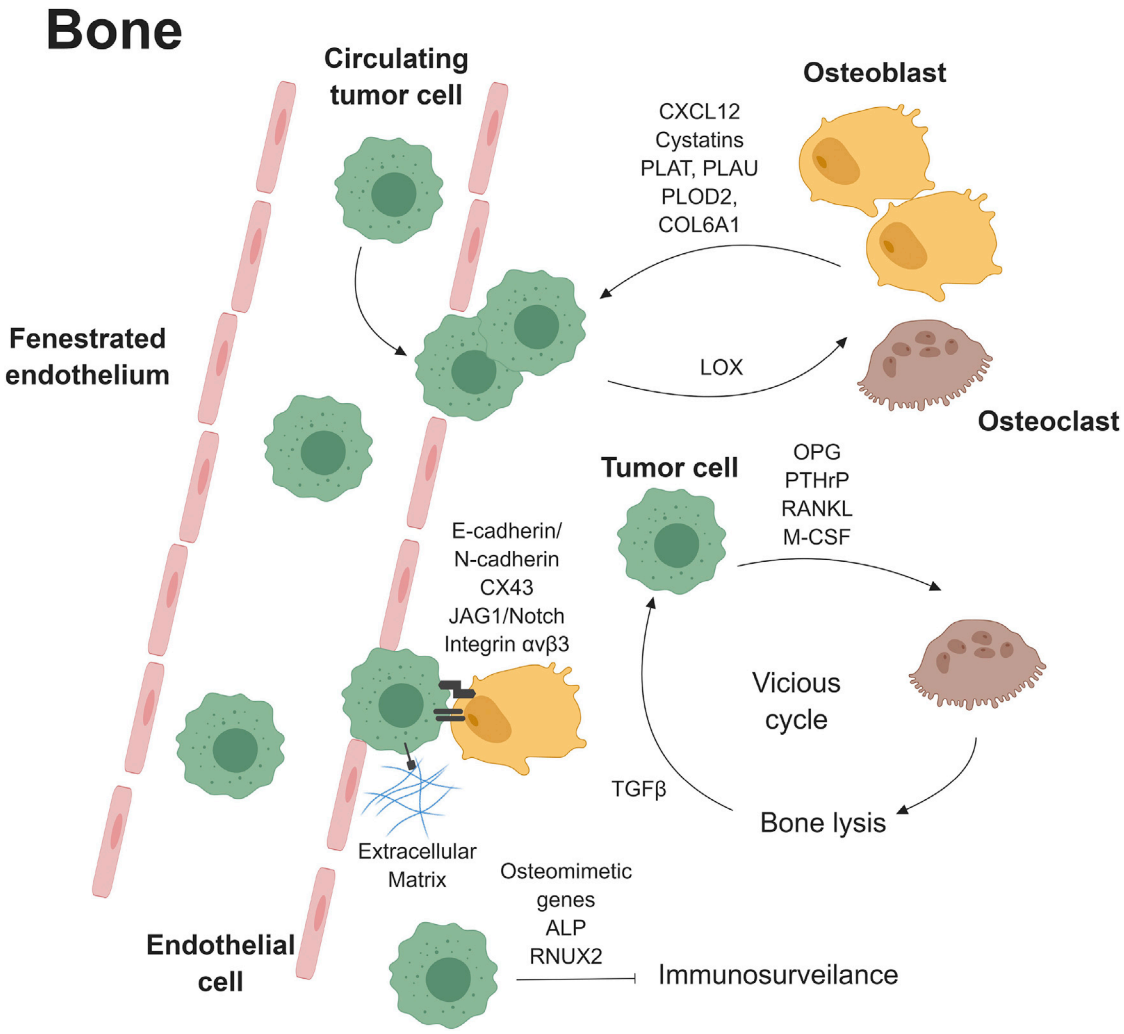

Figure 1. Bone-Specific Metastasis

The bone microenvironment (ME) secretome generated by osteoblasts, osteoclasts, or other cells may promote bone metastasis, while tumor cells can produce factors such as LOX to induce pre-metastatic niche formation. Interactions between tumor cells and osteoblasts through adherens junctions (via E-cadherin/N-cadherin, JAG1/Notch) and gap junctions (via CX43) also facilitate bone metastasis. Colonizing tumor cells express osteoblast-specific markers such as ALP and RUNX2 to escape immunosurveillance. In addition, tumor cells secrete factors promoting bone turnover to induce osteolysis, which in turn produces factors to stimulate tumor growth, creating a "vicious cycle."

micro-endoscopic multiphoton imaging over several months, provides evidence for an exceptionally dynamic vasculature in bone marrow (Reismann et al., 2017). These results suggest potential overlap of or interconversion between different niches, which may affect the cellular fates of bone metastatic seeds. Precise mapping of heterogeneous cancer, endothelial, and osteogenic cells will be required to delineate the co-evolution of different niches and the consequent impact on metastasis progression.

Osteomimicry. The selection of bone ME may drive the development of "osteomimcry," i.e., metastatic cancer cells may evolve to resemble bone cells. Both breast and prostate cancers can express osteoblast-specific markers including alkaline phosphatase (ALP) and Runt-related transcription factor 2 (Runx2), as well as other factors involved in bone turnover including osteoprotegerin, PTH-related peptide (PTHrP), RANKL, and macrophage colony-stimulating factor (M-CSF) (Rucci and Teti, 2010). Similarly, tumors can also express bone matrix proteins such as osteocalcin, sialoprotein, osteopontin, and osteonectin to mimic osteoblast activity (Huang et al., 2005; Rucci and Teti, 2010), enabling cancer cells to directly foster osteoclast maturation without osteoblasts. This may be essential in advanced osteolytic metastasis with a decreased osteogenic population. This process can be altered by microRNAs (miRNAs) such as miR218, which regulate the expression of osteomimetic genes in breast cancers with metastatic properties and high Wnt signaling (Hassan et al., 2012). Osteomimicry can be mediated by osteomimetic genes (Knerr et al., 2004). For instance, endothelin-1 was shown to be a bone-induced factor that drives osteomimicry in breast cancer (Bendinelli et al., 2014). More molecular and cellular mechanisms are reviewed in-depth elsewhere (Rucci and Teti, 2010).

Seed Pre-selection and Pre-metastatic Niche. Bone-tropism may already arise in primary tumors. Previous studies suggest

that cancer-associated fibroblasts contribute to creating a cytokine environment resembling the bone marrow, thereby selecting cancer cells that are more fit to colonize the bone ME even before dissemination. This is termed ''seed pre-selection'' and may explain why gene expression profiles of primary tumors can be used to predict bone metastasis (Zhang et al., 2009, 2013). Even before their arrival, tumor cells can induce the formation of a ''pre-metastatic niche,'' a supportive ME in distant organs that is conducive to their survival, attachment, invasion, immune evasion, and outgrowth (Peinado et al., 2017). Hypoxic tumors, for example, can recruit bone marrow cells to pre-metastatic sites through secretion of lysyl oxidase (LOX) (Erler et al., 2009). Other factors, including miRNAs, can also promote pre-metastasis niche formation. For example, cancer-derived miR25-3p exosomes were found to promote pre-metastatic niche formation through recruitment of hematopoietic progenitor cells (HPCs) and induction of vessel permeability and angiogenesis (Zeng et al., 2018).

Liver Tropism

Liver is one of the favored distant metastatic sites for solid tumors such as breast cancer, lung cancer, and gastrointestinal cancers (Table 1). It receives a dual blood supply from the hepatic portal vein and hepatic arteries and has a much lower sinusoid blood pressure gradient (Kumar et al., 2008; MacPhee et al., 1995). This unique architectural feature allows CTC access and facilitates their attachment to the sinusoidal endothelium for seeding. For example, the blood circulation of the colon and proximal rectum is drained through the hepatic portal system, while the blood of the distal rectum goes to the lung. This vascular organization correlates with the fact that colorectal cancer prefers liver metastasis with lung as the second favored metastatic site (Riihimäki et al., 2016a, 2016b). Indeed, a greater number of colorectal CTCs are trapped in the liver than in the peripheral blood (Denève et al., 2013). Furthermore, the endothelial layer of the liver sinusoid is fenestrated, which may be more permissive to extravasation, as compared to the well-organized endothelial wall and basement membrane in other organs (Figure 2) (Nguyen et al., 2009).

Genes and Pathways Specifically Implicated in Homing and Colonization to Liver. Systematic studies have identified tumor intrinsic factors favoring liver organotropism. By transcriptional profiling of breast cancer metastases, Kimbung et al. identified a 17-gene liver metastasis-selective signature (Kimbung et al., 2016). Of note, the majority of these genes are ECM genes involved in cadherin and integrin signaling pathways. In addition, citrullination of the ECM by colorectal cancer cell-derived peptidylarginine deiminase 4 (PAD4) is essential for the growth of liver metastasis, consistent with the finding that inhibition of PAD4 altered EMT markers and diminished metastasis (Table S2). Furthermore, the direct binding and interaction between cancer cells and hepatocytes also play a role in liver tropism (Mook et al., 2003; Tabariès et al., 2012). For instance, claudin-2 is prevalent in breast cancer liver metastases but not in bone or lung metastases, and it is essential for cancer cell-hepatocyte interactions and liver metastasis (Table S2).

Pre- and Pro-metastatic Niches in Liver. Liver metastasis depends on the formation of both pre- and pro-metastatic niches. Various circulating factors from cancer cells, particularly in the form of exosomes, can help establish the pre-metastatic niche. For example, exosomes from pancreatic ductal adenocarcinoma (PDAC) cells enriched in macrophage migration inhibitory factor (MIF) can activate Kupffer cells, inducing secretion of TGFβ. TGFβ triggers hepatic stellate cells to produce fibronectin, which promotes recruitment of bone marrow-derived macrophages that induce liver-specific metastasis (Costa-Silva et al., 2015). Exosome proteomics of several tumor models has identified different exosomes that establish the pre-metastatic niches in different organs. Specifically, Kupffer cells in the liver absorbed PDAC-secreted exosomes expressing integrin αvβ5 and released pro-inflammatory S100A8, resulting in liver tropism. Targeting integrin αvβ5 inhibited liver metastasis (Hoshino et al., 2015).

In addition, liver resident cells can promote the formation of pro-metastatic niches, which support the outgrowth of DTCs. For instance, hepatocytes coordinate myeloid cell accumulation and fibrosis within the liver to direct the formation of a pro-metastatic niche through IL-6-STAT3-serum amyloid A1 and A2 (SAA) signaling (Lee et al., 2019). Hepatic stellate cells, upon activation, secreted growth factors and cytokines such as PDGF, HGF, and TGFβ to promote ECM degradation, which stimulates angiogenesis and inhibits the immune response, establishing a permissive ME for tumor cells (Van Den Eynden et al., 2013; Kang et al., 2011).

Lung Tropism

Lung is another frequent metastatic site in cancers such as breast, melanoma, and thyroid (Table 1). The physiology of the lung makes it ideal for colonization and metastasis. The broad surface area and numerous capillaries provide opportunities for cancer cells to adhere, extravasate, and colonize. At the same time, the endothelial layer in the lung has tight junctions between endothelial cells and an intact basement membrane, thus representing a more restrictive barrier for extravasation as compared to bone and liver (Figure 3). One strategy adopted by tumor cells to traverse the endothelial wall and basement membrane is to induce the formation of discrete foci of vascular hyperpermeability by increasing focal adhesion kinase (FAK)/E-selectin and MMP9 expression in lung endothelial cells (Hiratsuka et al., 2002, 2011).

Tumor Intrinsic Factors for Lung Metastasis. Many cell-intrinsic factors associated with lung metastasis are present in primary tumors, suggesting, as discussed above for other metastatic sites that lung tropism may be pre-selected. Transcriptomic profiling of metastatic and non-metastatic breast cancer cells generated a list of 54 genes implicated in lung metastasis. The list includes secreted protein acidic and cysteine-rich (SPARC), vascular cell adhesion molecule 1 (VCAM1), angiopoietin-like 4 (ANGPTL4), ID1, and Tenascin C (TNC) (Minn et al., 2005). Many of these tumor-intrinsic factors disrupt vascular endothelial cell-cell junctions increased the permeability of lung capillaries and facilitated the trans-endothelial passage of tumor cells (Table S3). For instance, melanoma-derived SPARC promoted lung metastasis through inducing vascular permeability and extravasation in an endothelial VCAM1-dependent manner (Tichet et al., 2015). TGFβ in breast tumors induced ANGPTL4 to facilitate extravasation (Padua et al., 2008). Other factors may be involved in cell proliferation and survival (Table S3). For example, ID1 expression is selectively enriched in breast cancer lung metastasis and mediates lung colonization and sustained cell proliferation (Gupta

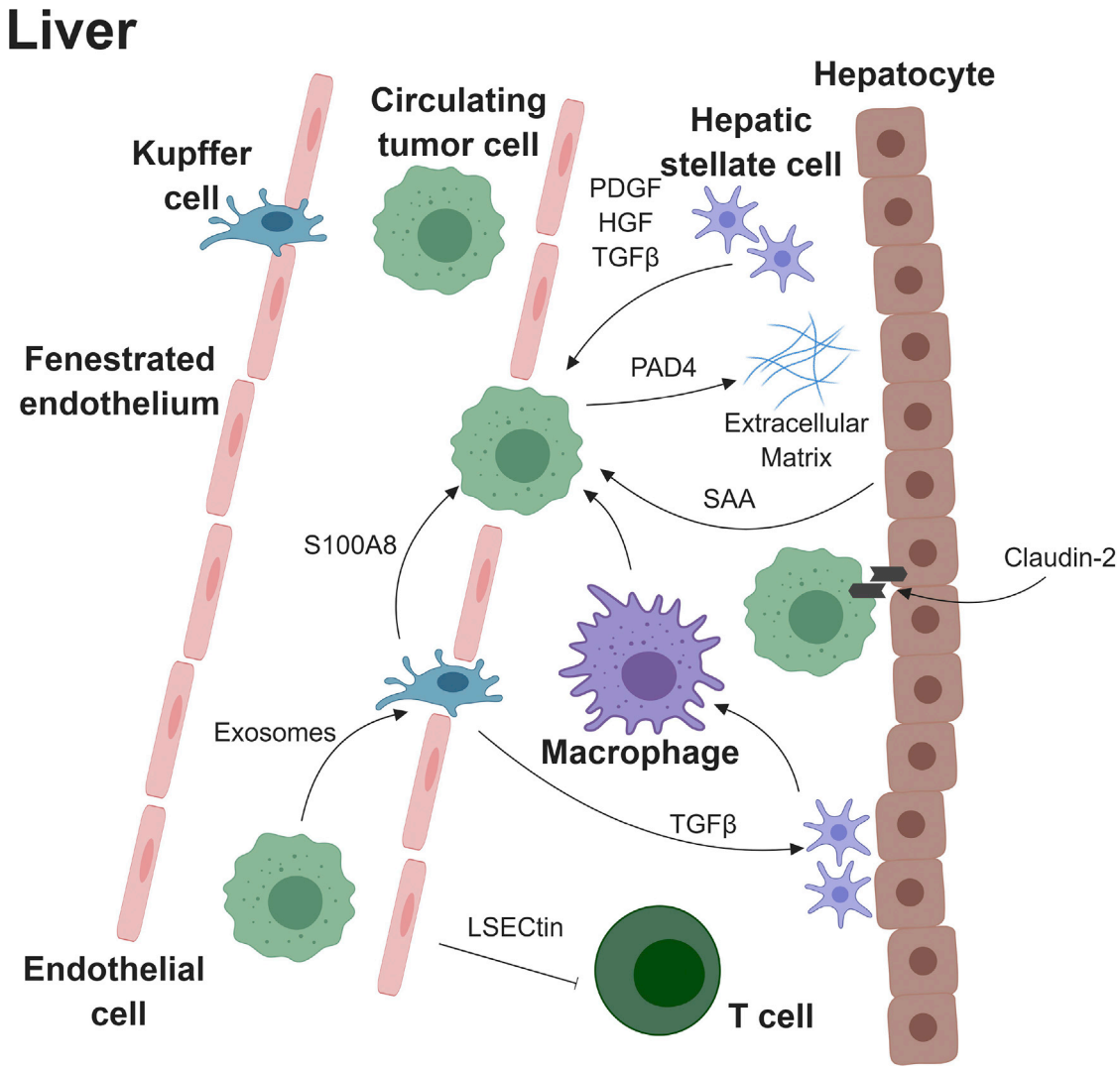

Figure 2. Liver-Specific Metastasis

Hepatocytes directly interact with tumor cell enriched in claudin-2 to promote liver metastasis. They also promote the formation of a pro-metastatic niche though secretion of serum amyloid A1 and A2 (SAA). Hepatic stellate cells can produce PDGF, HGF, and TGFβ to induce liver metastasis. Tumor cells secrete exosomes, which are taken up by Kupffer cells. Integrin αvβ5-enriched exosomes stimulate Kupffer cells to produce pro-inflammatory S100A8, whereas MIF-enriched exosomes trigger Kupffer cells to secrete TGFβ, which activates hepatic stellate cells, inducing liver-specific metastasis. LSECtin generated by sinusoidal endothelial cells also facilitates metastasis by inhibiting the T cell immune response.

et al., 2007). TNC, an ECM protein of stem cell niches, is produced by breast cancer cells that infiltrate the lung to increase stem cell-related signaling such as the Notch and WNT pathways to initiate metastasis (Oskarsson et al., 2011).

Extracellular Vesicles (EVs) and Pre-metastatic Niche in the Lung. Similar to liver metastasis, cancer cells also actively secreted EVs, including exosomes, to build the pre-metastatic niche in the lung (Table S3). Liu et al. found that both lung cancer and melanoma-derived exosomal RNAs activate toll like receptor 3 (TLR3) signaling in alveolar type II cells to induce chemokine secretion and recruit neutrophils to build up the pre-metastatic niche (Liu et al., 2016). Melanomas can also produce EVs to downregulate interferon alpha and beta receptor subunit 1 (IFNAR1) and IFN-inducible cholesterol 25-hydroxylase (CH25H) in normal cells to promote the formation of a pre-metastatic niche enriched with CD11b+ myeloid clusters and fibronectin deposits (Ortiz et al., 2019). In addition, breast cancer cells secreted integrins α6β4- and α6β1-positive exosomes, which in turn enhanced pro-inflammatory S100A4 expression in lung-resident fibroblasts to establish a pre-metastatic niche and promote lung metastasis (Hoshino et al., 2015). Furthermore, Keklikoglou et al. found that chemotherapy-elicited breast cancer EVs were enriched in annexin A6 (ANXA6), a Ca2+-dependent protein that promoted NF-κB-dependent endothelial cell activation, C-C motif chemokine ligand 2 (CCL2) induction, and Ly6C+CCR2+ monocyte expansion in the pulmonary pre-metastatic niche to facilitate the establishment of lung metastasis (Keklikoglou et al., 2019).

Pro-metastatic and Metastasis-Suppressive Niches. Lung resident cells can establish a pro-metastatic niche for many types of

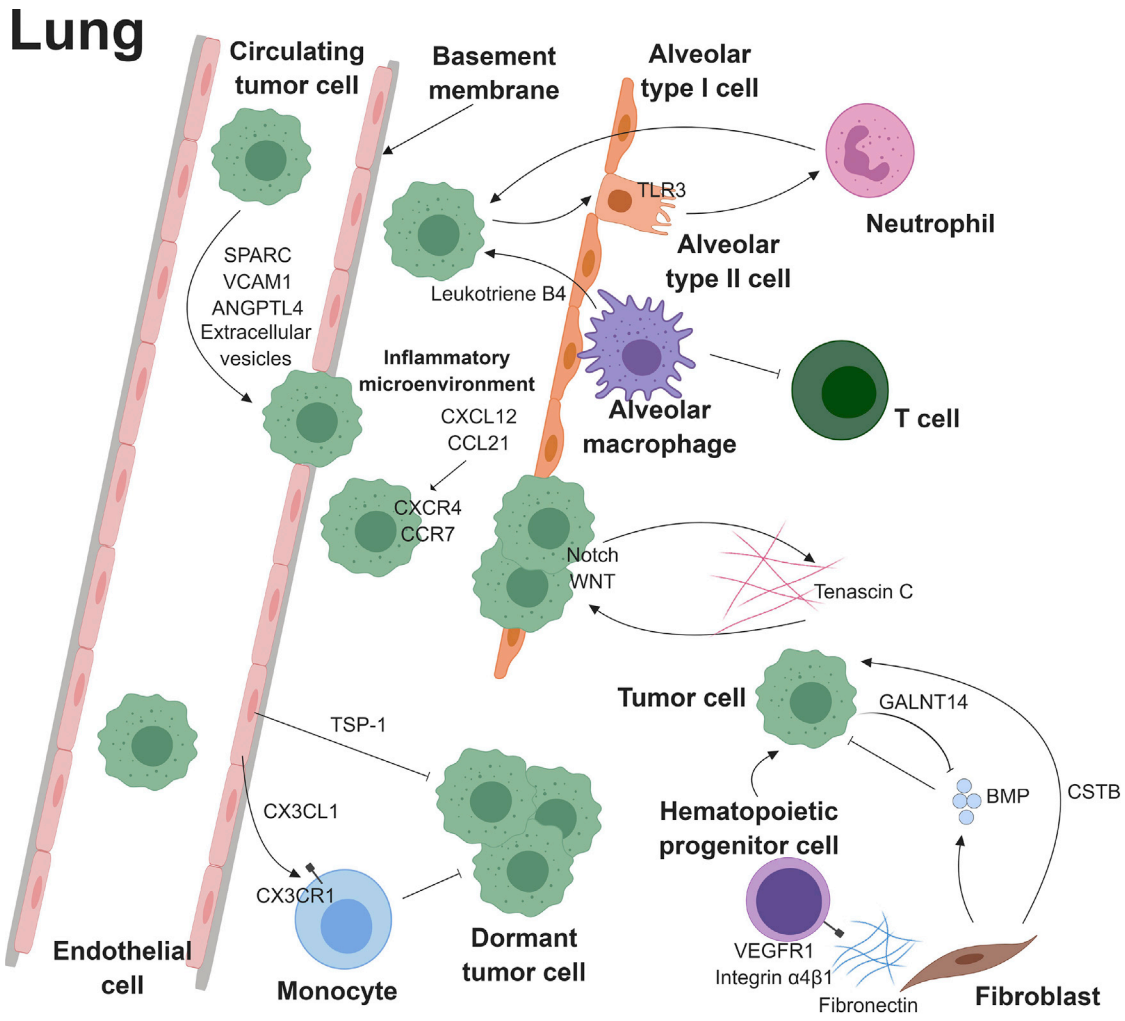

Figure 3. Lung-Specific Metastasis

Tumor-derived factors such as SPARC, VCAM1, and ANGPTL4, as well as EVs have been shown to be involved in tumor cell extravasation to the lung parenchyma. Tumor cells can activate TLR3 signaling in alveolar type II cells, which in turn recruit neutrophils and promote lung metastasis. Chemokines enriched in the lung such as CXCL12 and CCL21 recruit CXCR4- and CCR7-positive tumor cells. Alveolar macrophages can secrete the pro-inflammatory mediator Leukotriene B4 to suppress the T cell response and facilitate metastasis. Fibroblasts secrete CSTB to induce tumor cell survival. Also, fibronectin-enriched fibroblasts recruit VEGFR1 and integrin $\alpha4\beta1$ positive hematopoietic progenitor cells to facilitate metastasis. Tumor cells also produce tenascin C to initiate metastasis and GALNT14 to overcome dormancy signals from fibroblasts. TSP-1 secreted from endothelial cells inhibits tumor cells self-renewal, while CX3CL1 expression leads to recruitment of CX3CR1-positive patrolling monocytes, preventing metastasis.

cancer, e.g., they secrete abundant chemokines such as CXCL12 and CCL21, that direct breast cancer and melanoma cells that highly express CXCR4 and CCR7 to the lung (Müller et al., 2001). Fibroblasts also contribute to pro-metastatic niche formation in the lung. Liu et al. showed that fibroblast-secreted cathepsin B (CSTB) activated stearoyl-CoA desaturase 1 (SCD1), a critical modulator of cell proliferation, through the ANXA2 and PI3K/Akt/mTOR pathway and promoted metastatic colonization of melanoma cells. Besides modulating tumor cells, lung fibroblasts also generated fibronectin to recruit VEGFR1 and integrin $\alpha4\beta1$-positive bone marrow-derived HPCs to terminal bronchioles and bronchiolar veins, providing a permissive niche for incoming tumor cells (Kaplan et al., 2005).

In contrast to the pro-metastatic niche, several recent studies suggest that the lung also has metastasis-suppressive niches, which inhibit cancer cell proliferation (Altorki et al., 2019). For example, a perivascular niche expressing thrombospondin-1 (TSP-1)-induced sustained breast cancer cell quiescence, and this inhibitory effect was lost in sprouting neovasculature where active TGF-$\beta1$ and periostin were upregulated (Ghajar et al., 2013). The inhibitory effect of TSP-1 from the perivascular niche has also been identified in bone, suggesting a shared metastasis-suppressive mechanism among different organs (Ghajar et al., 2013). Lung fibroblast-derived bone morphogenetic proteins (BMPs) had an inhibitory effect on cancer stem cell self-renewal. Tumor cells, however, can secret polypeptide N-acetyl-galactosaminyltransferase 14 (GALNT14) to overcome this effect (Song et al., 2016). Furthermore, CX3C-chemokine receptor 1 (CX3CR1)+ monocytes, which were attracted by lung endothelial cell-derived CX3-chemokine ligand 1 (CX3CL1), in turn, recruited and activated natural killer (NK) cells to prevent lung metastasis (Hanna et al., 2015).

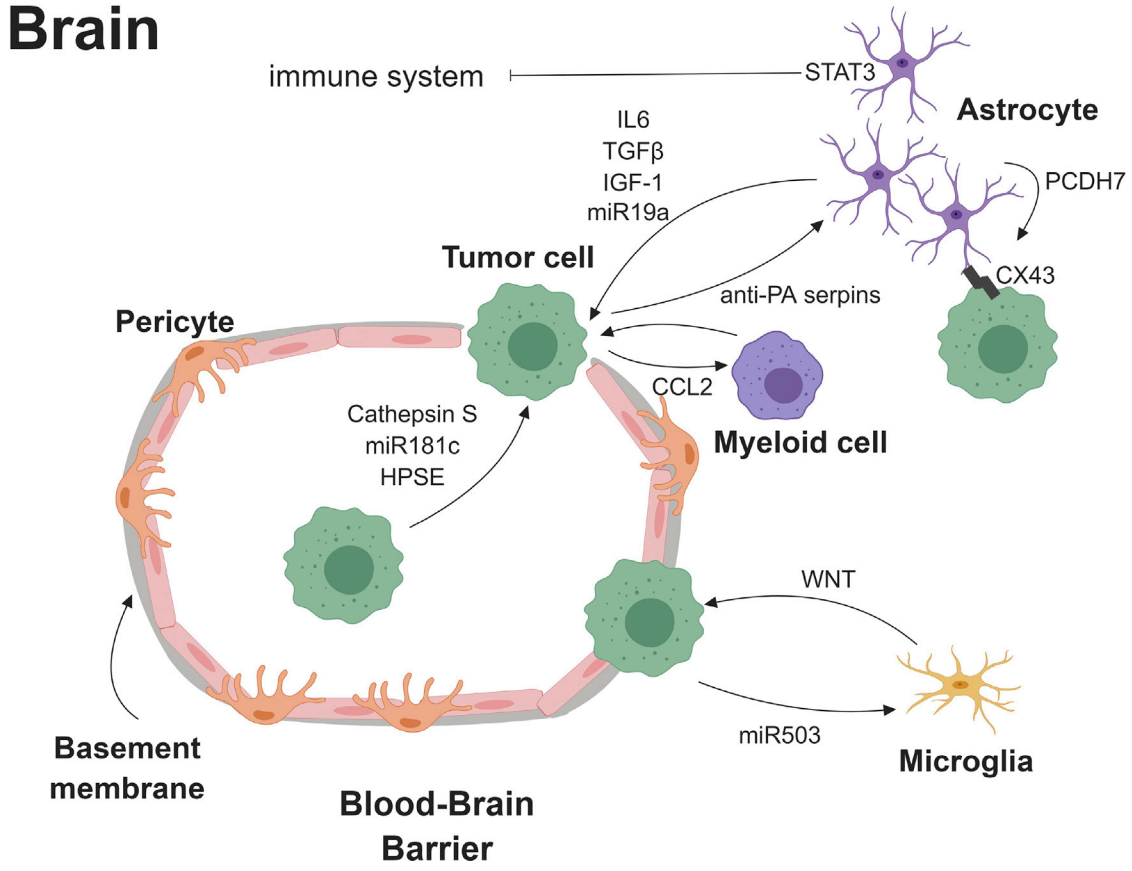

Figure 4. Brain-Specific Metastasis
Tumor cells colonizing the brain need to produce cathepsin S, miR181c-enriched EVs and HPSE to overcome the defense provided by the BBB. They also generate anti-plasminogen activator serpins to inhibit plasmin production from astrocytes to initiate metastasis. Astrocytes secrete many factors such as IL6, TGFβ, and IGF-1 that induce the growth of brain metastases. They also secrete miR19a-enriched exosomes, which inhibit PTEN expression and facilitate metastasis. Furthermore, gap junction characterized by CX43 between astrocytes and tumor cells and induced by PCDH7 stimulate brain metastasis. Exosomes enriched with miR503 induce M2 polarization of microglia, which can promote metastasis through WNT signaling. Of note, bone and liver contain a fenestrated endothelium in contrast to the smooth lining of the lung endothelium and BBB. Therefore, tumor cells develop different strategies to colonize at these different sites.

Brain Tropism

The majority of brain metastases come from lung cancer, breast cancer, and melanoma (Table 1). The brain is protected by the blood-brain barrier (BBB), which distinguishes it from other organs. The BBB is a continuous, non-fenestrated endothelium stitched together by tight junctions and supported by a basement membrane, astrocytes, and pericytes (Figure 4) (Weidle et al., 2016). These features protect the brain from being invaded by cancer cells. However, some tumors cells produce cathepsin S to proteolyze the junctional adhesion molecule (JAM)-B and facilitate the transmigration through the BBB (Sevenich et al., 2014). They may also release miR181c-enriched EVs to target PDPK1/cofilin-modulated actin dynamics to subsequently destroy the BBB (Tominaga et al., 2015). Once the extravasation of cancer cells is accomplished, the BBB may invert its roles from a barrier against cancer cells to a barrier against therapies. Indeed, the BBB protects brain metastatic tumor cells from many chemotherapeutic drugs (Valiente et al., 2018).

Brain-Tropic Genes. Brain metastasis-related genes and signaling pathways have been identified by different groups. A study of brain-seeking breast cancer cells in clinical samples identified 17 genes associated with brain metastasis. Among them, PTGS2 and the EGFR ligand HBEGF are commonly expressed in both pulmonary and cerebral metastases, whereas the α2,6-sialyltransferase ST6GALNAC5 is a brain metastasis-specific mediator (Bos et al., 2009). Similarly, a number of studies suggest brain metastatic cancer cells are capable of producing other factors to break through the BBB (Table S4). For example, heparanase, a potent proangiogenic enzyme, is overexpressed in breast cancer brain metastases and mediates transendothelial migration. Inhibition of HPSE by miR1258 suppressed brain metastasis (Zhang et al., 2011).

Special Contribution of Astrocytes to Brain Metastasis. The brain neural niche is primarily composed of neurons and glial cells (astrocytes, oligodendrocytes, and microglia) (Termini et al., 2014). Among them, astrocytes are the best-understood in brain metastasis. Astrocytes support neurons by secreting growth factors and cytokines, and this ability can be hijacked by tumor cells to facilitate metastasis (Valiente et al., 2018). For example, astrocyte-derived IL6, TGFβ, and IGF-1 increase tumor

cell proliferation (Seike et al., 2011; Sierra et al., 1997). Also, miR19a-enriched exosomes, derived from astrocytes and taken up by tumor cells, reduced the expression of PTEN, a major tumor suppressor. Astrocyte-specific depletion of miR19a, or blockade of astrocyte exosome secretion, rescued PTEN loss and suppressed brain metastasis. Furthermore, loss of PTEN and tumor cell-induced CCL2 expression recruited pro-metastatic myeloid cells (Zhang et al., 2015).

The interaction between cancer cells and astrocytes can promote tumor survival and protection from chemotherapy (Kim et al., 2011). Chen et al. found that cancer cell-derived protocadherin 7 (PCDH7) promoted the assembly of carcinoma-astrocyte gap junctions composed of CX43. These gap junctions transferred cyclic guanosine monophosphate–adenosine monophosphate (cGAMP) to astrocytes to activate the stimulator of interferon genes (STING) pathway and release inflammatory cytokines that support tumor growth and chemoresistance (Chen et al., 2016). Furthermore, plasmin from reactive astrocytes induces cancer cell death in a FasL-dependent manner and cleaves cancer cell-derived L1 cell adhesion molecule (L1CAM), a molecular required for vascular co-option and metastatic outgrowth. Both lung and breast cancer cells can produce serpins that inhibit astrocytes-derived plasmin in order to initiate metastasis in the brain (Valiente et al., 2014).

General Principles Underlying Organotropism

Despite the uniqueness of each distant organ, certain general principles underlying organotropism emerge. First, tropism may develop before dissemination, either through "seed pre-selection" or formation of a pre-metastatic niche. These mechanisms may explain why metastatic tropism often correlates with specific gene expression patterns in primary tumors. Second, specific chemotactic and adhesive factors may facilitate retention of DTCs in specific organs. This process may resemble immune cell homing to different peripheral organs. Third, distinct vascular structures in target organs establish specific requirements for cancer cell extravasation. For instance, the architecture of blood barriers in different organs may select for metastatic seeds with a different capacity for breaking down endothelial junctions. Fourth, unique resident cells, together with the secretome and ECM generated by these cells, determine the initial fate of cancer cells upon their arrival. It should be noted that even in the same tissue, different MEs may provide dramatically distinct milieus. Therefore, precise mapping of various niches in different organs is of critical importance, especially for understanding the early-stage colonization process. Fifth, the ability of cancer cells to hijack resident cells and remodel the ME determines if overt metastases can be established. During these initial interactions, cancer cells and the cancer-entrained microenvironmental cells may form vicious cycles that are difficult to terminate. Finally, all of the above processes involve specific interactions between "the seeds" (cancer cells) and "the soil" (ME), which can be dynamic and continuously evolving throughout the colonization process.

Passive Dissemination

Passive dissemination represents an alternative mechanism of metastatic spread in contrast to hematogenous or lymphatic metastasis, which requires the active steps of intravasation and extravasation. Passive dissemination includes the intraperitoneal dissemination of ovarian cancer and the direct invasion to adjacent organs by gastrointestinal cancers, including gastric, colorectal, and pancreatic cancers (Mikuła-Pietrasik et al., 2018). For example, ovarian tumor cells can disseminate to other organs in the peritoneal cavity through the passive movement of ascitic fluid (Lengyel, 2010; Mitra, 2016). Single or clusters of ovarian cancer cells detached from the tumor mass are carried by the peritoneal fluid and preferentially land on the abdominal peritoneum or omentum (Lengyel, 2010; Mitra, 2016). However, this process of intraperitoneal spread is not completely passive. EMT may still be required for the initial step of intraperitoneal dissemination (Lengyel, 2010). The ovarian tumor cells floating in ascites and in metastatic sites show reduced E-cadherin expression compared to cells in the primary tumors (Veatch et al., 1994). Downregulation of E-cadherin decreases intercellular adhesion and promotes the invasion of epithelial cells, which facilitates the detachment of ovarian cancer cells (Ellerbroek et al., 1999; Kalluri and Weinberg, 2009). The mesothelium is traditionally considered a passive barrier preventing the intraperitoneal spread of cancer cells (Davidowitz et al., 2014; Iwanicki et al., 2011). However, recent studies suggest that mesothelial cells may play an active role in promoting the progression of intraperitoneal metastasis (Kenny et al., 2014; Mikuła-Pietrasik et al., 2014, 2016a, 2016b). Specifically, TGFβ1 from ovarian cancer cells stimulates the secretion of fibronectin through the TGFβ receptor/RAC1/SMAD-dependent signaling pathway in mesothelial cells (Kenny et al., 2014). The increased deposition of fibronectin promotes the adhesion, invasion, proliferation, and metastasis of ovarian cancer cells (Kenny et al., 2014). Attachment of ovarian cancer cells to mesothelial cells also stimulates the secretion of IL-6 and IL-8, and thereby promotes the proliferation of ovarian cancer cells (Mikuła-Pietrasik et al., 2014). The frequency of intraperitoneal spread positively correlates with the age of ovarian cancer patients (Mikuła-Pietrasik et al., 2016a). Furthermore, aged mice are more susceptible to developing metastases in an ovarian allograft model (Loughran et al., 2018). Senescent peritoneal mesothelial cells may create a metastasis-favorable niche for ovarian cancer cells by releasing pro-cancerous factors and via direct cell-cell contact, which can be blocked by neutralization of p38 mitogen-activated protein kinases (MAPK) (Mikuła-Pietrasik et al., 2016a). In addition, the senescent mesothelial cells are reported to promote the neoangiogenesis by stimulating the production of pro-angiogenic factors such as C-X-C motif chemokine ligand 1 (CXCL1) and vascular endothelial growth factor (VEGF) by ovarian cancer cells (Mikuła-Pietrasik et al., 2016b).

Emerging Organotropism Mechanisms

Recent advances, which still await full validation, suggest additional organotropic mechanisms (Figure 5).

Metabolic Features of the Microenvironment

Significant metabolic changes accompany tumor progression and metastasis and raise the possibility of therapeutic targeting of metabolic pathways (Elia et al., 2018; Teoh and Lunt, 2018). However, it is often difficult to differentiate between pathways promoting metastasis and those simply supporting tumor growth. Tumor growth is usually characterized by rapid cell division, whereas metastasis involves migration, invasion, and survival, and these processes often exhibit divergent metabolic activities relative to the primary tumor. For example, in breast

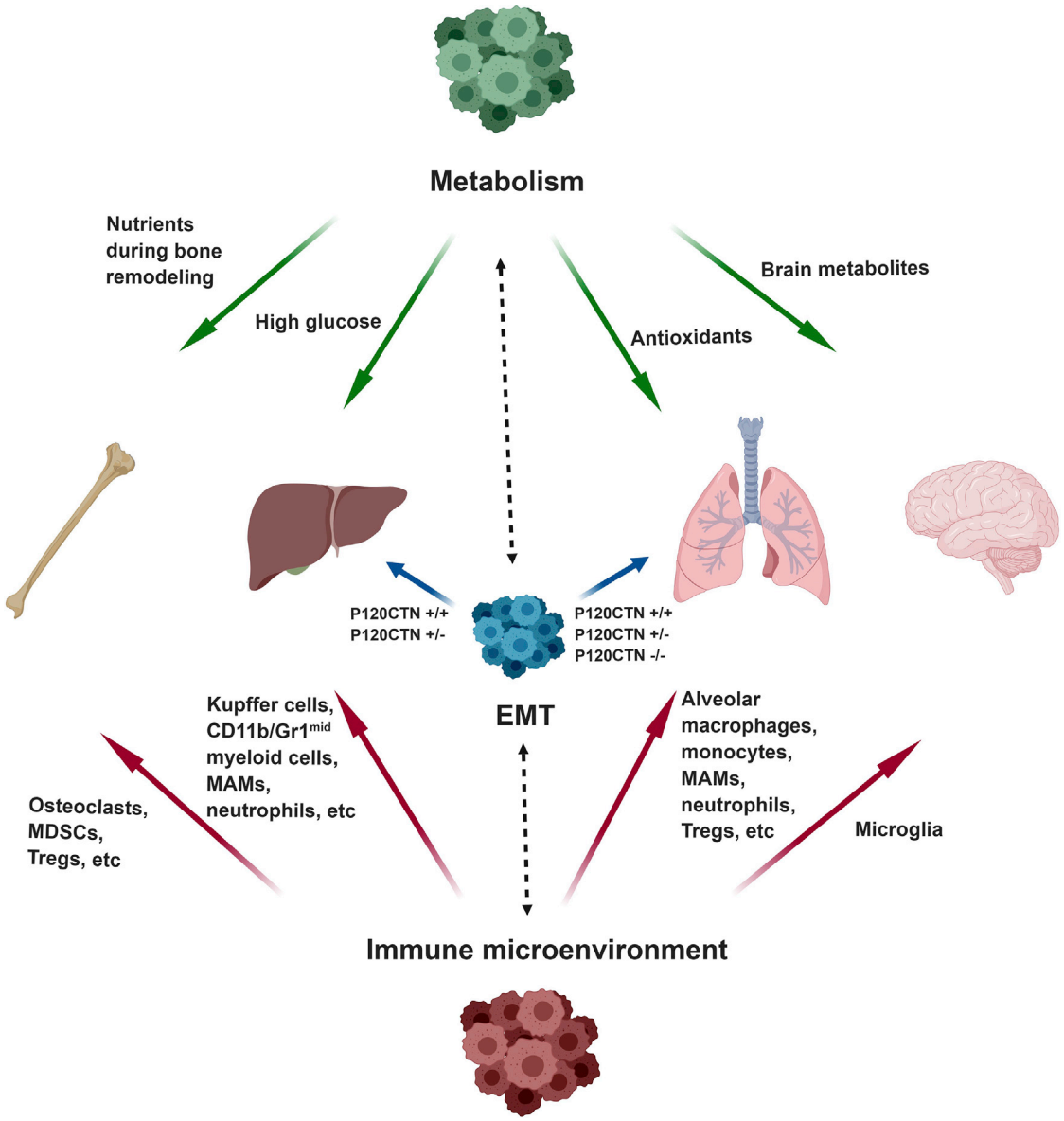

Figure 5. Emerging Facets of the "Seed and Soil" Hypothesis

Green, organotropic cancer cells possess distinct metabolic features. Bone metastases utilize nutrients released during bone remodeling, such as serine, glycine, glucose, and glycerol. Liver metastases are highly glycolytic and consume local glucose. Lung metastases develop antioxidant strategies for survival in the pro-oxidant lung environment. Brain metastases exploit brain metabolites such as acetate, glutamine, amino acids, and glutamine because of limited glucose resources. Blue, EMT regulates cancer organotropism. Liver metastases of pancreatic cancers require at least one copy of P120CTN, a stabilizer for membranous E-cadherin. Pancreatic cancer cells harboring homozygous P120CTN mutations can only metastasize to lung due to loss of E-cadherin. EMT may also contribute to organotropism through regulating metabolism and the immune microenvironment (ME). Red, different immune MEs regulate organ-specific metastasis. Bone metastasis is facilitated by osteoclasts, myeloid-derived suppressor cells (MDSCs), Tregs, and other bone-resident cells. Kupffer cells, CD11b/Gr1mid myeloid cells, metastasis-associated macarophages (MAMs), and neutrophils contribute to liver metastasis. Lung metastasis is regulated by alveolar macrophages, monocytes, MAMs, neutrophils, Tregs, and other cells. Microglia, the tissue-resident macrophages of the brain, promote brain metastasis.

cancer, the secretion of miR122 downregulates the level of pyruvate kinase isozyme M2 (PKM2), which in turn increases glucose availability in tumor cells but decreases glucose consumption of the ME niche. Interestingly, while overexpression of miR122 promotes metastasis to brain and lung, primary tumor growth is reduced (Fong et al., 2015). Similarly, while the fatty acid receptor CD36 drives metastasis in oral squamous cell carcinoma, melanoma, and breast cancer, the effect of CD36 on primary tumor growth is limited, suggesting a unique role of lipid metabolism in metastasis initiation (Pascual et al., 2017). Metabolic differences between primary tumors and metastases are further illustrated by differential regulation of mitochondrial metabolism. The downregulation of mitochondrial genes correlates with poor clinical outcomes across several cancer types in primary tumors (Gaude and Frezza, 2016). However, mitochondrial metabolism is paradoxically upregulated in metastatic breast cancer (Kim

et al., 2014). In this section, we will focus on organ-specific metabolic reprogramming of metastasis. More comprehensive insights into metabolic phenotypes in the general metastatic cascade have been reviewed previously (Elia et al., 2018; Luo et al., 2017; Pascual et al., 2018).

Only a very small minority of DTCs ultimately initiate the outgrowth of an overt metastasis (Obenauf and Massagué, 2015). A growing body of evidence suggests that metabolic interactions with the tumor ME indeed play an important role in metastasis propensity and progression. Upon colonization, survival of cancer cells usually requires adaptation to the metabolism of local tissues with respect to energy, nutrient, and oxygen availability (Schild et al., 2018). Consequently, cancer cells undergo a tissue-specific metabolic rewiring for colorization, survival, and outgrowth (Gaude and Frezza, 2016).

Bone. Most overt bone metastases are osteolytic in breast cancer but osteoblastic in prostate cancer. Upon the onset of bone resorption, metastatic metabolism may be rewired by nutrients released from the bone matrix, including serine, glycine, glucose, and glycerol (Shi et al., 2014). In contrast, metabolic reprogramming of osteoblastic metastasis is still poorly understood. Considering the recent finding that early-stage bone colonization of breast cancer often occurs in the osteogenic niche (Wang et al., 2015, 2018), an environment that is more "osteoblastic", it is possible that the metabolic activities of DTCs need to undergo a drastic change when transiting from microscopic osteogenic to macroscopic osteolytic metastases. And this change may represent a bottleneck in the escape from metastatic dormancy, a yet to be understood step in breast cancer metastasis.

Liver. The liver plays a central role in all metabolic processes in the body, especially for the equilibrium of glucose and fatty acid synthesis. Similar to the hypoxic glycolytic profile of local hepatic cells, liver metastases are also characterized by high glycolytic activity and reduction in mitochondrial metabolism (Dupuy et al., 2015). Additionally, Vriens et al. discovered an unconventional fatty acid desaturation pathway, involving sapienate biosynthesis in liver carcinomas, which further increased hepatic metabolic plasticity in cancer cells (Vriens et al., 2019). Furthermore, in hepatic metastasis of colorectal cancer, an enhanced fructose metabolism was observed via the upregulation of the enzyme aldolase B (ALDOB), hence providing extra fuel for metastatic outgrowth (Bu et al., 2018). Thus, as the metabolic center of the entire organism, the liver appears to provide a unique milieu enabling or forcing cancer cells to assume specific metabolic activities for their colonization.

Lung. Lung is the organ of respiration, meaning that local tissues are exposed to high oxygen levels. Accordingly, cancer cells metastasized to lung often develop antioxidant strategies to overcome oxidative damage and stress. For example, by upregulating peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC-1α), breast cancer cells drastically enhance mitochondrial biogenesis to counteract electron leakage and ROS generation (Lebleu et al., 2014). Further antioxidant strategies include the upregulation of peroxiredoxin 2 (PRDX2), a small antioxidant protein (Stresing et al., 2013), as well as the overexpression of dihydropyrimidinase-like 4 (DPYSL4), a protein facilitating oxygen consumption (Nagano et al., 2018). Exposure to oxygen in the lung ME may represent a contrast to bone, which is generally hypoxic. How this difference contributes to organ-specific metastasis will be an interesting question to tackle.

Brain. Brain tissues have the highest energy demands of all organs. Although glucose is the primary source of energy, other sources can also be utilized. In brain metastasis, when glucose is limiting, cancer cells display metabolic flexibility that enables them to exploit locally available nutrients such as acetate, glutamine, amino acids, and glutamine (Ebert et al., 2003). Brain metabolic mimicry can extend to catabolism of γ-aminobutyric acid (GABA). For example, some brain metastatic cells upregulate GABA receptors and transporters to better utilize GABA as an energy source for protein synthesis (Neman et al., 2014). How cancer cells adapt to the unique metabolic environment in the brain and acquire the ability to utilize brain-specific pathways remains to be further investigated.Overall, it is tempting to hypothesize that cancer cells evolve to resemble the metabolic phenotype of the relevant distal organ during colonization. Further investigations of tissue-specific metabolic rewiring of metastatic cells at different stages of colonization will be required to test this hypothesis. This is technically very challenging but will substantially enrich our knowledge of metastatic organ-tropism.

Immune ME and Organotropism

Recent breakthroughs in characterizing the tumor immune ME and cancer immunotherapy highlight the importance of immune cells in the formation of metastasis (Kitamura et al., 2015). Immunosuppressive cells not only help cancer cells evade immunosurveillance and improve their survival in the circulation, but also actively modify the ME of target organs to prepare a premetastatic niche (Liu and Cao, 2016; Peinado et al., 2017).

Different organs harbor unique tissue-resident immune cells that are involved in metastasis. Macrophages are the major type of tissue-resident immune cells and have been extensively studied in variable metastatic settings. For example, VCAM1-expressing micro-metastases were found to recruit integrin $\alpha4\beta1$-positive osteoclasts, tissue-resident macrophages in bone, to accelerate their micro- to macro-metastasis transition (Lu et al., 2011), indicating that enrichment of osteoclasts in the tumor ME contributes to cancer cell awakening and progression to macrometastasis. Kupffer cells are liver-specific tissue-resident macrophages, which exert phagocytic and cytotoxic activity toward DTCs (Kolios et al., 2006). However, they can also trap CTCs at the liver sinusoid, increasing their chances of colonizing the liver (Bayón et al., 1996). Early depletion of Kupffer cells increased metastatic burden, but depletion at a later stage of tumor growth decreased metastasis (Wen et al., 2013). The alveolar macrophages in lung are also a double-edged sword for CTCs. The tumoricidal activity of alveolar macrophages has been long recognized; however, recent studies suggest that these cells can also promote lung metastasis. Alveolar macrophages have been shown to suppress T cell responses and to generate the pro-inflammatory mediator Leukotriene B4 to facilitate lung metastasis (Nosaka et al., 2018; Sharma et al., 2015). Microglia are the tissue-resident macrophages in the brain. Their cytotoxic function induces cancer cell apoptosis (He et al., 2006). However, similar to other tissue-resident macrophages, microglia can also promote metastasis to their host organ. Pukrop et al. showed that microglia enhance invasion and

colonization of breast cancer cells in the brain via a WNT-dependent pathway (Pukrop et al., 2010). Also, in breast cancer cells, loss of the lncRNA XIST led to increased secretion of exosomal miR503, which induced M2 polarization of microglia to promote metastasis (Xing et al., 2018). Indeed, it appears that primary tumor, CTC and/or metastatic lesions are able to reeducate tissue-resident macrophages to promote metastasis progression in their host organs. Understanding the genes and signaling pathways implicated in this process and determining how to interrupt and reverse these processes will require further investigation. Furthermore, recent studies suggest that tissue-resident macrophages in different organs have different origins (embryonically derived and/or monocyte-derived), which may correlate with different functions (Epelman et al., 2014). Although macrophages from disparate origins may carry out similar functions, their epigenomic landscape remains distinctive and may influence their capacity to be educated by cancers.

Immune cells can also be systemically recruited by tumors to aid organ-specific metastasis, primarily because of their immunosuppressive functions and ability to establish the pre-metastatic niches. Below, we present examples of how these immune cells affect organ-specific metastasis.

Bone. The HSC niche, which is likely the niche harboring DTCs, may have immune privilege through recruitment of immunosuppressive cells. Sawant and colleagues found that bone marrow-derived plasmacytoid dendritic cells recruited myeloid-derived suppressor cells (MDSCs) and T regulatory cells (Tregs) to bone metastases of breast cancers to inhibit tumor-specific cytolytic CD8+ T cells and promote metastatic colonization (Sawant et al., 2012). In a breast cancer model, MDSCs were found in a greater number in bone metastases than in primary tumors and lung metastases (Bidwell et al., 2012). Silencing of interferon-7 (IRF7) mediated type I IFN signaling in breast cancer cells facilitated bone metastasis by further restricting immunosurveillance, and restoration of IRF7 significantly decreased MDSCs but increased CD8 T cells and NK cells, which inhibit bone metastasis (Bidwell et al., 2012). In addition, tumor-educated CD4+ T cells have been shown to promote bone metastasis by secreting RANKL and inducing pre-metastatic osteoclastogenesis (Monteiro et al., 2013). Indeed, one intriguing aspect of bone (and bone marrow) is the fact that it is where most immune cells are generated, but typically in immature or naive states. It is therefore conceivable that tumor-induced immune responses may be unique in the bone, although this is understudied and remains to be elucidated.

Liver. Liver organotropism is also mediated by specialized immune cells beside Kupffer cells. For example, Zhao et al. found that a myeloid cell subset (CD11b/Gr1 mid) recruited by a CCL2/C-C chemokine receptor 2 (CCR2) signaling pathway promoted colorectal cancer liver metastasis (Zhao et al., 2013). Both bone marrow-derived macrophages and neutrophils also help prepare the liver pre-metastatic niche (Costa-Silva et al., 2015). Metastasis-associated macrophages (MAMs) can secrete granulin to activate hepatic stellate cells. Upon activation, hepatic stellate cells develop into periostin-producing myofibroblasts, which produce a fibrotic ME that sustains metastatic tumor growth (Nielsen et al., 2016). The tissue inhibitor of metalloproteinases-1 (TIMP-1) recruits neutrophils to increase liver metastases in a CXCL12/CXCR4-dependent manner (Seubert et al., 2015). Neutrophils, in turn, induce the liver pre-metastatic niche through fibroblast growth factor 2-dependent angiogenesis (Gordon-Weeks et al., 2017). Neutrophils also play a role in liver-specific colonization by promoting lung cancer cell adhesion to liver sinusoids. Macrophage-1 antigen (Mac1) was found to be responsible for interactions between neutrophils and CTCs (Spicer et al., 2012). Importantly, the immune tolerance of liver is unique because of the liver's ability to metabolize a wide variety of xenobiotic compounds (Tiegs and Lohse, 2010). Therefore, in the liver, DTCs can take "shelter" from the immune system. For example, liver sinusoidal endothelial cell-derived lectin (LSECtin) inhibited the hepatic T cell immune response and enhanced cancer cell migration, thereby promoting colorectal cancer liver metastasis (Tang et al., 2009; Zuo et al., 2013).

Lung. The role of different immune cells in organotropism has been extensively studied in lung metastasis. For instance, CCL2 produced by cancer cells and myeloid cells recruits inflammatory monocytes and CD206+/Tie2+ macrophages to pulmonary metastatic sites and pre-malignant lesions, respectively, to orchestrate breast cancer lung metastasis. Inflammatory monocytes secrete VEGF to facilitate tumor cells extravasation, whereas CD206+/Tie2+ macrophages downregulate E-cadherin and cancer cell adhesion to promote dissemination and intravasation (Linde et al., 2018; Qian et al., 2011). Macrophages recruited to lung metastasis can also bind to cancer cells through an interaction with integrins α4/VCAM1, which subsequently activate Ezrin-PI3K/Akt signaling to prolong the survival of cancer cells in the lung (Chen et al., 2011). Neutrophils support lung metastasis initiation by secreting leukotrienes, which selectively expand clones with high tumorigenic potential (Wculek and Malanchi, 2015). In addition, neutrophil extracellular traps (NET) induced by inflammation are required for dormant cancer cells to awake in the lung. Mechanistically, two NET-associated proteases, neutrophil elastase, and matrix metalloproteinase 9 cleaved laminin and subsequently induced integrin α3β1 signaling-dependent proliferation of dormant cancer cells (Albrengues et al., 2018). MDSCs recruited to breast tumors secrete the cytokines TGF-β, IL-6, and IL-23, which induce the accumulation of T-helper cell 17 (Th17). Th17 cells, in turn, secrete IL-17, which promotes further recruitment of MDSCs and lung metastasis (Novitskiy et al., 2011). CCR4+ Treg cells have also been implicated in lung metastasis by inhibiting NK cells through secreting β-galactoside-binding protein (βGBP) (Olkhanud et al., 2009). Of note, loss of RON kinase promotes a CD8+ T cell response that specifically inhibits the outgrowth of lung metastasis (Eyob et al., 2013). Unlike other organs, the lung is constantly exposed to the external environment. On one hand, pathogens need to be immediately eliminated, entailing an efficient and rapid innate immune response. On the other hand, these responses need to be tightly regulated to avoid excessive inflammation and tissue damage. As such, the lung provides a distinctive immune ME for metastasizing tumors. Further studies are needed to investigate how the tightly controlled inflammatory milieu influences metastatic colonization in the lung.

Brain. The brain may be another organ with an immunoprivileged ME due to the protection of the BBB (Peinado et al., 2017). However, increasing evidence indicates that the interaction between BBB and circulating immune cells is not uniform, and various types of immune cells can indeed cross the BBB

in different physiological and pathological conditions (Schwartz et al., 2013). Moreover, brain metastatic progression may disrupt the BBB to allow increased immune cell influx. However, the roles of immune cells in brain metastases remain at this time poorly understood.

In summary, the immune landscape of metastasis reflects the combined effects of tumor-intrinsic pathways (leading to variable secretomes), the local environment (tissue-resident immune cells and vascular barriers), and the systemic host environment. Primary tumors may alter the local environment in distant organs before metastatic seeding occurs. The local environment may critically influence the seeding process, when cancer cells are still few in number and likely under stress in the foreign milieu. As cancer cells successfully survive and progress in distant organs, they may evolve the ability to educate local immune cells and to systemically recruit new immune cells, which may promote further progression. Because of different immune contexts, specific organs may impose different selective pressures on metastatic cancer cells and entail organ-specific mechanisms. Comparative analyses of the immune ME of metastases in different organs will likely provide novel insights into these mechanisms.

EMT and Organotropic Metastasis

EMT is critical for metastasis as discussed elsewhere (Lambert et al., 2017; Yeung and Yang, 2017). However, a recent study also implicates EMT in metastatic organotropism (Reichert et al., 2018). In this study, deletion of P120CTN, a stabilizer for membranous E-cadherin, in metastatic PDAC models shifted the metastatic burden from the liver to the lung. Additional experiments showed that liver but not lung metastasis required at least one copy of p120ctn. Furthermore, lung organotropism of the p120ctn homozygous PDAC cells could be shifted to the liver by transfection of p120ctn isoform 1A. These phenotypes were associated with P120CTN-mediated E-cadherin expression and epithelial integrity as demonstrated by the selective pressure for E-cadherin-positive liver metastasis and E-cadherin-deficient lung metastasis in the Cdh1 heterozygous PDAC mice.

In addition to direct regulation of organotropism, EMT pathways may affect organ-specific metastasis through regulating cancer cell metabolism. By analyzing metabolic gene expression in 978 human cancer cell lines, Shaul et al. found that mesenchymal cell lines share 44 upregulated metabolic genes. Among them, dihydropyrimidine dehydrogenase (DPYD), a pyrimidine-degrading enzyme, was highly expressed upon EMT induction (Shaul et al., 2014). SNAIL, a key transcriptional repressor of EMT, reprogramed glucose metabolism in breast cancer by repressing phosphofructokinase (PFKP) and fructose-1,6-bisphosphatase 1 (FBP1), which pushed the glucose flux toward the pentose phosphate pathway and granted cancer cell survival advantages (Dong et al., 2013; Kim et al., 2017). Therefore, EMT may interact with metabolic reprogramming during cancer cell metastasis and contribute to organotropism.

Tumor-infiltrating immune cells such as tumor-associated macrophages (TAMs) and MDSC can promote EMT (Chockley and Keshamouni, 2016). On the other hand, accumulating evidence suggests that EMT can regulate the tumor immune ME. Snail-induced EMT increased the number of Tregs and reduced the number of dendritic cells, which promoted melanoma metastasis to the lung (Kudo-Saito et al., 2009). In lung cancer cells, the EMT activator ZEB1 repressed the EMT suppressor miR200, thereby relieving the miR200-mediated inhibition of PD-L1, an immune-checkpoint protein, thus leading to CD8 T cell immunosuppression and metastasis (Chen et al., 2014). Furthermore, EMT-induced modulation of E-cadherin and cell adhesion molecule 1 (CADM1) regulated NK cell-mediated metastasis-specific immunosurveillance (Chockley et al., 2018). The link between EMT and tumor immune ME suggests that EMT could regulate organ-specific metastasis through modulating immune cells.

Recent studies suggest that epithelial plasticity of tumor cells cannot be classified simply as either epithelial or mesenchymal. Instead, cancer cells may possess a spectrum of intermediate states (Pastushenko et al., 2018). This hybrid epithelial-mesenchymal state has been termed "partial EMT" (P-EMT) and may be regulated by epithelial protein internalization, in contrast to the transcriptional repression of epithelial genes in complete EMT (Aiello et al., 2018; Grigore et al., 2016). P-EMT PDAC cells preferably traveled as clusters of CTCs, whereas PDAC cells that underwent a complete EMT invaded as single CTCs (Reichert et al., 2018). Furthermore, CTC clusters showed specific DNA hypomethylation at binding sites of several EMT transcription factors, such as OCT4, NANOG, SOX2, and SIN3A (Gkountela et al., 2019). However, the formation of CTC clusters required cell adhesion components such as plakoglobin that are also epithelial markers (Aceto et al., 2014), further supporting the P-EMT status of CTC clusters. Given the importance of epithelial plasticity in organotropism, the role of P-EMT and CTC clusters in organ-specific metastasis needs to be considered and warrants further investigation.

Conclusions and Perspectives

With a few exceptions, the "seed and soil" hypothesis still provides a conceptual framework for our understanding of metastasis organotropism. Within this framework, an increasing number of molecular and cellular mechanisms have been discovered in recent studies. The specificity of the metastatic process in each organ is largely based on the unique ME, which consists of unique resident cell types, ECM, and secretomes. Cancer cells that either evolve fitness or adapt to the local ME exhibit tropism toward the particular organ. Their "fitness" or adaptability are typically encoded by epigenomic programs and manifested by the expression or activation of specific genes and pathways that allow cancer cells to exploit the specific aspects of the ME in which they reside, to endure a hostile ME, and to ultimately remodel the ME to fuel aggressive colonization.

One significant advance in our understanding of the metastatic ME is the "rediscovery" of various "niches" within each organ with highly organized spatial structures, specialized resident cells, and often well-defined physiological functions, as previously shown for other processes such as hematopoiesis. Examples include the perivascular and osteogenic niches in the bone, which appear to dictate different cellular fates of DTCs. To better understand the organization and cell interactions within these microenvironmental niches, analyses at the single-cell level, combined with cutting-edge microscopic techniques will be needed.

The recent renaissance of tumor immunology and metabolism have extended our definition of "seed and soil" to new dimensions. Pioneering studies have begun to reveal how metabolism

of metastases may differ from that of primary tumors. Considering the vastly varying oxygen levels, acidity, and metabolite profiles of different organs, it would not be surprising to find unique tumor characteristics matching organ-specific metabolic environments. These environments may play particularly important roles in the early stages of colonization, before cancer cells acquire the ability to remodel the host environment. Similarly, baseline immune profiles may represent another major difference among organs, which may be reflected in the types of cancer cells able to invade the organ. The combination of specific invading cancer cells and the local immune landscape may lead to engagement of different "defense" systems, which may be utilized to tailor immunotherapies.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.devcel.2019.04.012.

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Senesce to Survive: YAP-Mediated Dormancy Escapes EGFR/MEK Inhibition

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Therapeutic resistance is a major challenge in cancer treatment. In this issue of *Cancer Cell*, Kurppa et al. demonstrated that a senescence-like state enables lung cancer cells to survive dual inhibition of EGFR and MEK. This was mediated by the YAP/TEAD pathway, which drives epigenomic reprogramming and EMT to counteract apoptosis.

Therapeutic resistance is a primary concern in cancer treatment. Designated targets often acquire mutations that make cancer cells less vulnerable. The Yes-associated protein (YAP) and Transcriptional enhancer factor TEF-1 (TEAD) are conserved downstream effectors of the Hippo pathway that are involved in cancer progression and therapeutic resistance in a variety of cancers. In a study published in this issue of *Cancer Cell*, Kurppa et al. (2020) provided a deeper understanding on how YAP signaling epigenetically reprograms lung cancer cells, allowing them to escape cell death through dormancy. The implication of this finding opens opportunities to evaluate other cancers with similar phenotypes.

With an estimated 142,000 deaths in 2019, lung cancer has the highest rate of mortality in cancer. Over 80% of lung cancers are classified as non-small cell lung cancer (NSCLC). Oncogenic drivers of NSCLC include EGFR and KRAS mutations. EGFR, also known as ErbB1, is a member of a family of 4 receptor tyrosine kinases (ErbB1, ErbB2, ErbB3, and ErbB4), which can be activated by multiple growth factors, including EGF and TGF-α. As a transmembrane protein, EGFR dimerizes upon ligand binding and subsequently transactivates downstream effectors involved in cell proliferation, survival, and migration (Avraham and Yarden, 2011). EGFR tyrosine kinase inhibitors (TKIs) are the standard of care for advanced NSCLC. The clinical outcome has not been very successful, as resistance almost certainly occurs due to mutations abolishing drug binding or activating alternative pathways (Kobayashi et al., 2005). Multiple approaches have been implemented to overcome resistance, including (1) co-targeting of the extracellular domain and tyrosine kinase domain using anti-EGFR monoclonal antibodies and EGFR TKIs, respectively, and (2) preventing EGFR pathway reactivation by pharmacologically inhibiting key downstream effectors such as RAF, MEK, or ERK. These approaches significantly delay resistance to EGFR but do not reduce recurrence (Tricker et al., 2015), implicating alternative pathways.

The Hippo signaling pathway plays a central role in regulating cell fate, proliferation, and apoptosis, mainly by repressing the oncogenic transcription factors YAP and TAZ (Harvey et al., 2013). Previous studies identified YAP as a resistance factor in multiple cancers, including NSCLC, and revealed that the co-inhibition of YAP and MEK can lead to synthetic lethality in tumors harboring BRAF and RAS mutations (Lin et al., 2015). Here, Kurppa et al. (2020) further dissect the mechanisms of resistance following EGFR/MEK co-inhibition in NSCLC bearing EGFR mutations.

Kurppa et al. (2020) observed a senescence-like phenotype as a survival strategy for cancer cells following EGFR/MEK combination treatment. Intriguingly, the senescent phenotype appeared to be reversible, as supported by live imaging and lineage tracing. The authors then identified a strong epigenetic alteration driven by YAP/TEAD in response to EGFR/MEK signaling inhibition. Mechanistically, YAP promotes survival of cancer cells through activation of an epithelial-to-mesenchymal transition (EMT) process, which in turn suppresses the pro-apoptotic factor, Bcl2-modifying factor (BMF). Importantly, the function of YAP/TEAD was verified in xenografts and clinical specimens. The authors also found that cooperation between YAP, TEAD, and the EMT marker SLUG was necessary to repress the pro-apoptotic factor BMF. These results corroborate previous findings on the importance of YAP/TEAD signaling in therapeutic resistance (Yu et al., 2018). Taken together, this novel YAP/TEAD/SLUG/BMF axis represents a connection between epigenetic reprogramming, EMT, and survival as a response to therapeutic stress and demonstrates stress-induced adaptation via activation of alternative pathways in cancer (Figure 1).

It remains debatable whether cancer cells can exploit senescence-related processes to enter a dormancy state and endure environmental stresses. By definition, dormancy implies reversibility—dormant cancer cells should maintain the potential to "wake up." This is seemingly contradictory to the general notion of senescence, which was initially thought of as a permanent cell-cycle arrest (Hanahan and Weinberg, 2000). However, many recent studies argued that senescence can be reversed, and the status of senescence mediators (e.g., p16, p21, RB, and ARF) may change after the senescence process is triggered, allowing cells to resume proliferation (Kuilman et al., 2010). In fact, multiple additional aspects,

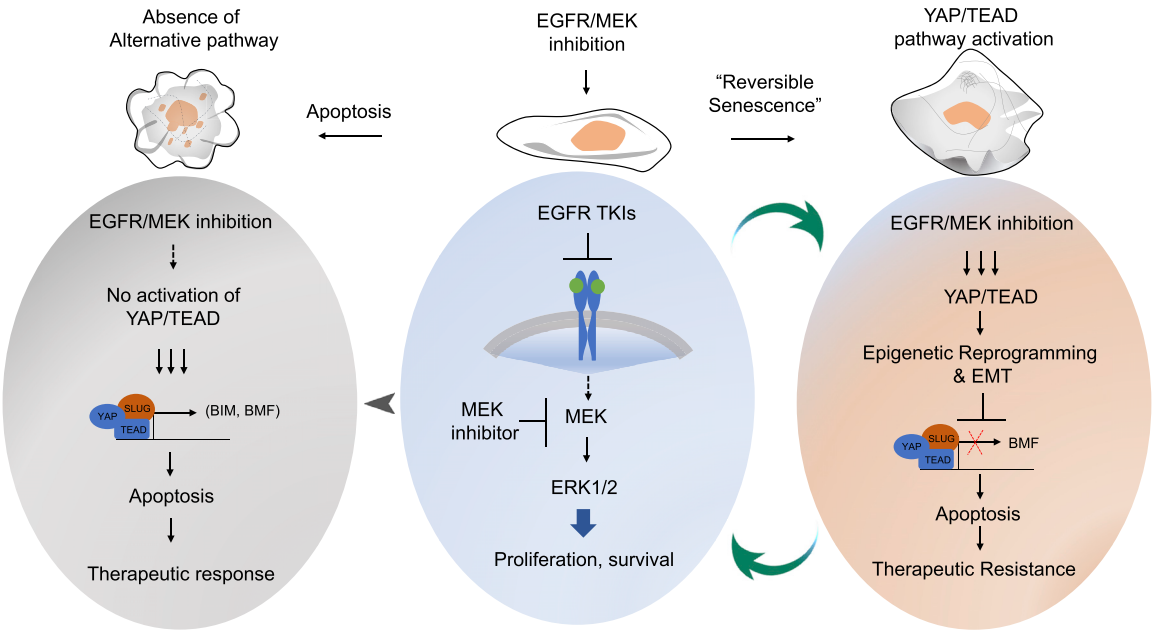

Figure 1. Reversibility of YAP/TEAD-Induced Senescence as a Survival Mechanism to Dual Inhibition of EGFR and MEK in Advanced Non-small Cell Lung Cancer (NSCLC)

Complete inhibition of EGFR/MEK signaling prevents pathway reactivation by maintaining ERK1/2 inactive (middle panel). As a result, cells undergo apoptosis, leading to massive cell death (left panel). Surviving cells adopt a senescent phenotype, which allows them to escape cell death (right panel). This process is mediated by the activation of an alternative pathway, YAP/TEAD signaling, which triggers epigenetic reprogramming and EMT to oppose apoptosis. In the absence of therapeutic pressure, the senescent process is reversed and gives rise to a new "naive" population.

including DNA content, metabolic state, cell-cycle regulators, and lysosomal stress markers, have been characterized to distinguish senescence from transient quiescence (Sharpless and Sherr, 2015). Findings in this work may stimulate further studies to refine our understanding of senescence and cancer cell dormancy, especially under therapeutic settings.

Tumor heterogeneity is a major challenge in cancer treatment. In this study (Kurppa et al., 2020), it was shown that the selection for resistant cells was not clonal, as the cells remain sensitive after recovering from therapeutic stress. Interestingly, although the increased expression of YAP following EGFR/MEK inhibition was observed in most cells, the survival was still limited to a rare subset. The authors used a bar-coding experiment to rule out genetic selection as the major determinant of cell survival. However, the process may not be completely stochastic either—it is still possible that the dynamic cellular states (e.g., stemness and hybrid EMT) are acting cooperatively to drive the "selection." It will be interesting to further dissect the regulation of the YAP/TEAD/SLUG/BMF pathway at a single-cell level and examine its interactions with other factors that may together dictate the fates of individual cells.

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Trends in Cancer

CellPress REVIEWS

Review

Bone Metastasis: Find Your Niche and Fit in

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Metastasis to bones is determined by both intrinsic traits of metastatic tumor cells and properties appertaining to the bone microenvironment. Bone marrow niches are critical for all major steps of metastasis, including the seeding of disseminated tumor cells (DTCs) to bone, the survival of DTCs and microscopic metastases under dormancy, and the eventual outgrowth of overt metastases. In this review, we discuss the role of bone marrow niches in bone colonization. The emphasis is on complicated and dynamic nature of cancer cells–niche interaction, which may underpin the long-standing mystery of metastasis dormancy, and represent a therapeutic target for elimination of minimal residue diseases and prevention of life-taking, overt metastases.

Highlights

Bone metastasis is determined by cancer cell-intrinsic traits as well as their interactions with the unique bone microenvironment.

The bone metastasis niche undergoes dynamic changes during different stages of bone colonization including dormancy, reactivation, and outgrowth.

Various cell types including osteoblasts, osteoclasts, and immune cells reside in and regulate the bone metastasis niche by direct cell–cell contact and paracrine signaling.

Parallels with the hematopoietic stem cell niche may aid our understanding of the bone metastasis niche.

Bone may harbor a reservoir of tumor cells for further metastatic dissemination.

Therapies targeting cancer–niche interactions may eradicate micrometastases and prevent overt metastases.

Common Metastatic Traits versus Bone Tropism

The survival of cancer patients has been significantly improved with advances in early detection and treatments. However, the spreading of cancers to distant organs, known as cancer metastasis, is often incurable and is the major cause of death in cancer patients. The skeleton is one of the most common metastatic sites, particularly in breast and prostate cancers [1]. Bone metastasis is most likely associated with a unique set of skeletal complications, including bone pain, pathologic fractures, hypercalcemia, and spinal cord compression, which lead to a reduced quality of life or even death in these patients [2]. Similar to metastasis to other organs, bone metastasis is a process of multiple steps, including local migration and invasion, intravasation into the circulation, systemic dissemination, arrest and extravasation into bone marrow, survival under dormancy, reactivation, and ultimate outgrowth (Figure 1) [3]. Thus, bone metastasis is partly regulated by processes and pathways commonly related to metastasis in general, such as **epithelial–mesenchymal transition** (see Glossary), **cancer stem cell**-like properties, and escape of **immunosurveillance** in metastatic tumor cells [4]. By contrast, organ tropism of metastasis has also been noticed since over a century ago. About 90% and 70% of patients dying of prostate and breast cancer, respectively, show evidence of skeletal involvement at autopsy [5,6]. Tumor cells with certain molecular characteristics also appear to more commonly metastasize to the bone. Such metastatic predilection to bones can be intuitively explained by the '**seed and soil**' hypothesis, according to which the metastatic tumor cells must interact with the unique bone environments to establish successful metastasis [7].

Origins of Bone Metastatic Traits

Bone is unique for its mineral content, matrix composition, extreme rigidity, high concentration of extracellular calcium, hypoxia, and acidic pH [8]. Such a unique environment imposes specific requirements on metastatic seeds (Box 1). In addition to these physiochemical properties, bone matrix is also enriched for matrix proteins, cytokines, and growth factors, including ligands for integrins, insulin-like growth factors (IGFs), transforming growth factor beta proteins (TGFβs), fibroblast growth factors, platelet-derived growth factors, and bone morphogenetic proteins (BMPs), all of which have been proposed to regulate bone metastasis either directly or indirectly [9–12]. For example, TGFβ is deposited into the mineral bone by osteoblasts and could be released and activated by osteoclasts during bone remodeling

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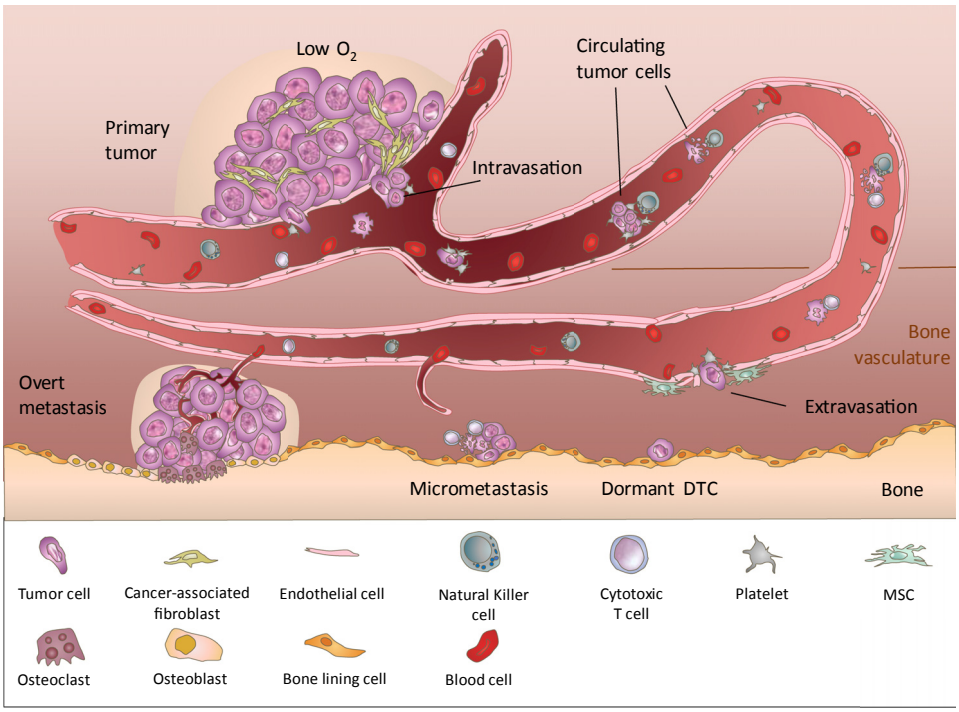


Figure 1. Steps of Bone Metastasis. Bone metastasis is a multiple step and lengthy process. Facilitated by the epithelial–mesenchymal transition and the primary tumor microenvironment (e.g., hypoxia and cancer-associated fibroblasts), invasive tumor cells may intravasate into the blood vessel as single circulating tumor cells (CTCs) or CTC clusters. While in circulation, CTCs may aggregate with platelets to survive against physiochemical pressure and immunosurveillance. After arrival at the bone marrow vasculature, CTCs attach and adhere to the bone marrow endothelium via intercellular adhesion, and extravasate into bone marrow parenchyma. Tumor cells may subsequently enter a dormant state for a prolonged period, until they are reactivated under favorable conditions to form micrometastasis. The progression of micrometastases into overt metastases is limited by neoangiogenesis and immunosurveillance mechanisms. Overt metastases in bone commonly leads to abnormal bone growth or resorption, both of which reduce bone strength and increase the risk of bone fracture. Abbreviations: DTC, disseminated tumor cells; MSC, mesenchymal stromal/stem cells.

[1,8,10]. Multiple important functions of TGFβ in bone metastasis have been implicated including induction of local invasion and angiogenesis, suppression of the antitumor immune system, regulation of tumor dormancy, and initiation of osteolytic vicious cycle [11,13].

The complex bone environment is selective for cancer cells with specific molecular traits. It is known that **bone tropism** is linked to sex steroid hormones and related pathways. Specifically, bone is the predominant metastatic site for estrogen receptor alpha (ERα)-positive (ERα+) luminal breast cancer, whereas ERα-negative (ERα–) basal-like breast cancer tends to metastasize to lungs and brains but less commonly to bones [14]. How such different organ tropism develops in different subtypes of breast cancer is largely unknown. We showed in a previous study that Src hyperactivation is tied to latent bone metastasis by supporting cancer cell survival in the bone microenvironment [15]. The mutual activation between Src and ERα may provide one possible mechanism of the bone tropism of ERα+ breast cancer [16,17]. However, Src inhibitors, such as dasatinib, exhibited very limited clinical efficacies on heavily pretreated metastatic breast cancer [18–20], suggesting that additional pathways may drive further metastasis progression and therapeutic resistance. By comparison, the role of androgen–androgen receptor (AR) axis is

Glossary

Bone tropism: the propensity of certain tumors, including prostate and breast cancers, to metastasize to the skeleton system.
Cancer stem cell: a rare subset of cancer cells within tumors with self-renewal, differentiation, and *in vivo* tumor initiation capacities.
Disseminated tumor cells: tumor cells that have left the primary tumor, survived the circulation, and finally landed on the parenchymal layer of distant organs.
Ductal carcinoma *in situ*: the earliest form of breast cancer. The cells have become malignant but have not spread out of the milk duct of the breast. It is considered a noninvasive disease.
Epithelial–mesenchymal transition: a conversion of polarized epithelial cells toward a mesenchymal phenotype, which is considered the initiating step of metastasis. Tumor cells may lose cell–cell adhesion and gain migrative and invasive capacity during the transition.
Hematopoietic stem cells: a population of progenitor cells that can differentiate into all types of blood and immune cells.
Immunosurveillance: a process by which the immune system identifies and clears foreign pathogens and precancerous and cancerous host cells. Tumor cells must escape immunosurveillance to successfully colonize in distant organs.
Mesenchymal stromal/stem cells: a population of multipotent stromal cells giving rise to the specialized cells in skeletal tissues, including osteoblasts, chondrocytes, fibroblasts, and adipocytes. They are commonly perivascularly located.
Minimal residue disease: refers to the persistent tumor cells in cancer patients without symptoms or signs of disease after systemic treatments, including circulating tumor cells and disseminated tumor cells.
Osteoblasts: bone cells originating from mesenchymal stem cells, which produce bone matrix and form new bones.
Osteoclasts: a type of large multinucleate cells that break down bone tissues. They belong to the myeloid lineage, and are derived from HSCs.

Box 1. Adaptation to the Physiochemical Properties of Bone Environment

Bone is unique in many aspects, and tumor cells must adapt to the unique environment to successfully colonize in bone.

Firstly, the inorganic phase of bone is mainly composed of the mineral hydroxyapatite (HA) nanocrystals. HA crystals have been shown to promote the mitogenesis and secretion of matrix remodeling enzymes of breast cancer cells [146]. Furthermore, the bone-tropic subline of MDA-MB-231 cells showed increased secretion of pro-osteoclastic IL-8 in an HA-rich environment, which may therefore contribute to the vicious cycle [147]. In a recent study, the skeletal sites with less mature HA were found to be more likely to develop bone metastasis in a mouse model of breast cancer [148].

Secondly, the extracellular bone matrix is enriched with type-I collagen, OPN, and BSP. Elevated expressions of OPN and BSP have been observed in cancers with bone tropism [31]. Overexpression of integrin αvβ3 in breast cancer cells facilitates tumor cell adhesion to collagen and BSP, and is associated with an increased metastatic propensity to bone [149]. Moreover, inhibition of a collagen receptor, discoidin domain receptor-1 (DDR1), has been shown to reduce the homing and colonization of lung cancer cells to bone [150].

Thirdly, the high mineralization content of bone gives rise to its rigidity. Bone-tropic cancer cells can benefit from the high stiffness by enhancing the production of PTHrP and the response of Fyn kinase to a rigid matrix [151–153].

Fourthly, the bone environment is imbued with calcium ions. A high calcium level has long been proposed to activate pathways that promote the survival and metastatic capacity of tumor cells [66,154–156]. In addition, calcium plays a key role in osteolytic vicious cycle by stimulating the production of PTHrP in tumor cells [157].

Fifthly, the bone marrow is known to be a highly hypoxic environment. As a result, the overexpression of hypoxia-induced factor 1α (*HIF1α*) was observed in two-thirds of bone metastases [158]. Hypoxia is known to modulate multiple steps of bone metastasis, including the premetastatic niches, dormancy, and osteolytic vicious cycles [37,50,159].

Lastly, the acidic environment of bone may be further exemplified by abnormal metabolic activities of metastatic cancer cells [160,161]. An acidic milieu increases bone resorptive activity, which may contribute to the osteolytic vicious cycle [162].

Osteomimicry: a phenomenon of bone-tropic tumor cells to express genes and to secret matrix proteins that are commonly restricted to bone cells.

Seed and soil: a hypothesis raised by Stephen Paget to explain the metastatic preference to specific organs. The metastatic tumor cells (the seed) must interact with the distant organ microenvironment (the soil) to establish the successful colonization.

elusive in bone metastasis of prostate cancer. The metastases of prostate cancer are mainly androgen independent but remain dependent on the activity of AR [21]. Meanwhile, activation of Src family kinases, which are also directly activated by AR, was found to correlate with the occurrence of metastases in hormone-independent prostate tumors [22], suggesting the potential involvement of AR–Src pathway in prostate cancer bone metastasis.

High-throughput profiling speeds up the discovery of genetic and epigenetic traits associated with bone metastasis [15,23–28]. Intriguingly, many such traits appear to be also relevant for regulation of bone hemostasis. For instance, the bone metastatic subline of MDA-MB-231 breast cancer cells highly overexpresses interleukin-11 (IL-11), which is produced by bone marrow stromal cells and osteoblastic cells to stimulate osteoclastogenesis [23,29]. Human prostate cancer bone metastasis specimens and bone-tropic sublines of LNCaP cells show increased expression of bone-related matrix proteins, including osteopontin (*OPN*), bone sialoprotein (*BSP*), and osteocalcin [30,31]. Overexpression of BSP in brain-tropic subline of MDA-MB-231 cells could redirect them to form metastases in the bone [32]. Thus, bone tropism may be related to the capacity of tumor cells expressing such bone-related factors, known as **osteomimicry** [30,33]. In addition to osteomimetic properties intrinsic to tumor cells, other components of primary tumors may also display bone stromal cell characteristics and therefore influence the selection of bone metastatic seeds. For instance, **mesenchymal stromal/stem cells** (MSCs) and cancer-associated fibroblasts in breast tumors create a bone marrow-like environment through secreting C–X–C motif chemokine ligand 12 (CXCL12) and IGF1 [34]. The Src-hyperactive bone metastatic seeds are therefore preselected from the heterogenous tumor mass because of their survival advantage conferred by enhanced PI3K–AKT activity in this bone marrow-like environment [34]. This mechanism, termed 'metastasis seed-preselection', may help explain why organ-specific metastatic outcome is associated with some gene expression features of primary tumors [34,35].

It is now clear that the distant organs could be remotely conditioned by the primary tumors ahead of metastatic spread. The tumor-derived factors and extracellular vesicles prepare a 'fertile soil' for future metastasis, which is now commonly termed as 'premetastatic niche' [36]. A special premetastatic niche mechanism was recently described in the context of bone colonization of ERα– breast cancer cells. Activation of hypoxia-induced factor 1α (HIF1α) by the hypoxic environment in primary tumors stimulates the secretion of lysyl oxidase (LOX), thereby enhancing the bone resorptive activity of osteoclasts and creating a favorable, osteolytic lesion for arriving tumor cells [37]. In another study, Engblom *et al*. [38] found that lung cancer leads to increased osteoblastic activity and bone mass, even without the presence of bone metastases in mouse model and human patients. This in turn triggers the production and infiltration of a specific population of tumor-promoting neutrophils into the primary tumors [38]. It remains to be determined if such increase of bone mass could be observed in other types of cancer and whether it contributes to bone metastasis. Organ tropism can also arise through extracellular vesicles or exosomes shed from primary tumors. The metastatic distribution of melanoma cells to bones was increased in mice pretreated with exosomes derived from highly metastatic melanoma cells as compared to mice treated with exosomes from poorly metastatic melanoma cells, suggesting that tumor-derived exosomes may educate bone environment to facilitate later metastatic colonization [39]. Furthermore, exosomes with distinct expression of specific integrins may target certain organs, where they mediate organ-specific development of the premetastatic niche, yet the exosomes specifically targeting bones have not been characterized so far [40].

The Course of Bone Colonization

Compared to the metastatic diseases in other organs, bone metastases appear to follow a somewhat unique course. First, the dissemination of tumor cells to bone marrow can be an early event, even preceding the formation of invasive primary tumors. In mouse models, cytokeratin-positive epithelial tumor cells were detected in the bone marrow in the premalignant phase of breast tumors [41–43]. This was further supported by the presence of **disseminated tumor cells** (DTCs) in the bone marrow of patients with **ductal carcinoma *in situ*** and pathologically localized prostate cancer [41,44,45]. Second, DTCs are present in the bone marrow of patients with various types of cancers, including ovarian, gastric, and colorectal cancers [46–48], yet clinically overt bone metastases were rarely observed in some of these cancer types [2], suggesting that dissemination to bone may not be sufficient for the onset of bone metastases. Lastly, despite their early dissemination, a significant proportion of overt bone metastases are detected very late, even decades after the diagnosis of primary tumors [9,10,15]. This is in contrast to the relatively fast progressing visceral metastases, and suggests a long dormancy period in bone metastases.

Clinical evidence and experimental models support that DTCs may remain in a quiescent state for years in the bone marrow [49]. This cellular dormancy is characterized by cell cycle arrest at the G_0 phase and a lack of proliferating markers [49,50]. Multiple pathways have been implicated in regulating quiescence of DTCs, such as PI3K–AKT signaling, TGFβ2–p38 axis, hypoxia signaling, BMPs, and WNT family [50–54]. Notably, DTCs also express surface markers of stem cell-like population, such as CD44, EpCAM, and ALDH1 [55–57]. Moreover, DTCs are resistant to the adjuvant therapies [58–60]. These suggest that DTCs share common features with cancer stem cells.

The dormant DTCs could re-enter cell cycle and form multicellular micrometastases when the microenvironment becomes growth permissive. At this stage, although the tumor cells may be dividing, tumor mass could be steady or only slowly growing due to a balance between cell

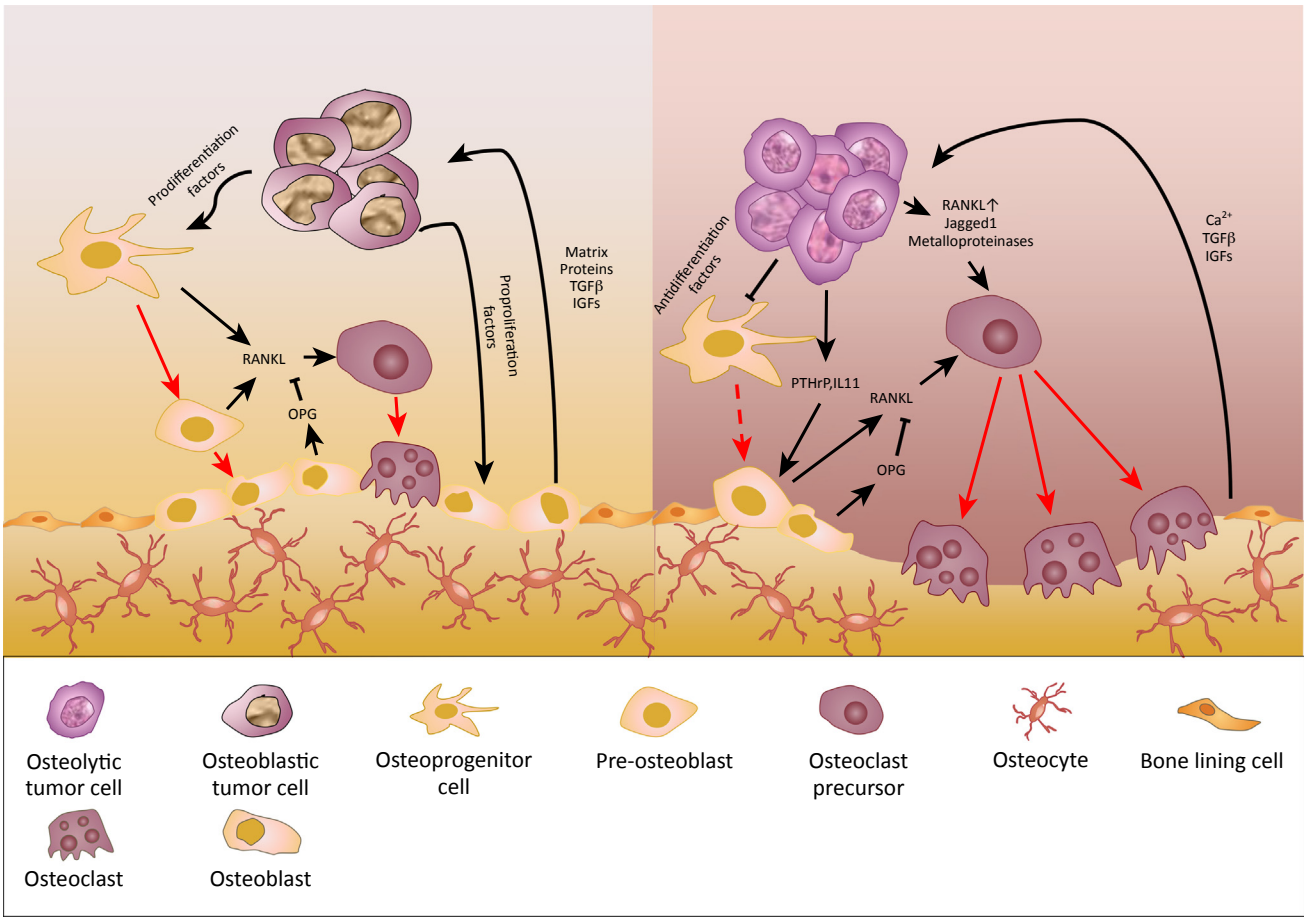
proliferation and apoptosis [49,50]. Hurdles for tumor expansion may include active immunosurveillance and lack of angiogenic support [49,50]. This clinically undetectable stage of metastasis was less characterized until recently. Several molecules and pathways have been identified by us and other groups to play important roles at this stage of bone metastasis, including VCAM-1–integrins, adhesion and gap junction proteins, *IRF7*, *MSK1*, and nephronectin [61–66]. For instance, we found that bone micrometastases predominantly reside in an osteogenic niche [62]. Tumor cells utilize E-cadherin to interact with N-cadherin on niche cells, and perturbation of this heterotypic junction can block niche-conferred growth advantage [62].

The more advanced stages of bone metastasis have been extensively studied, which involve the accelerating positive feedback between tumor and bone environment. Different molecular mechanisms drive the two different types of bone metastasis: osteoblastic (bone-forming) metastasis and osteolytic (bone-resorbing) metastasis (Figure 2). While prostate cancer predominantly generates osteoblastic lesions, metastases from breast cancer, multiple myeloma, and lung cancer mostly result in osteolytic lesions in bone [1,10,67]. Despite the distinction, most bone metastases are mixtures of both types and exhibit simultaneously enhanced **osteoclast** and **osteoblast** activities [1,10,67], probably due to the tight coupling between osteoclast and osteoblast functions.

Overt metastases at distant organs are often incurable because of therapeutic resistance [68]. This resistance might arise in much earlier stages of colonization. Several studies have suggested that DTCs could persist after systemic adjuvant treatments [58–60]. Importantly, the presence of DTCs in bone marrow not only predicts skeletal metastasis but also metastases in other organs [69–72]. As the primary tumors and affected lymph nodes are mostly resected after diagnosis, these persisting **minimal residue diseases** in bone might be the major source of seeds fueling metastases to distant sites. This idea is supported by the following evidence. First, metastatic tumor cells can be recirculated and colonize second distant organs in mouse models [73,74]. Second, in the clinic, many patients die of metastases at multiple organs rather than single-site metastasis [75]. Third, whole-genomic sequencing of multiple metastases from the same cancer patient indicates the seeding of bone metastasis to other metastatic sites [76–78]. However, many questions remain unanswered. For instance, what proportion of metastatic lesions are seeded from other metastases? Does such metastasis-to-metastasis spreading utilize distinct mechanisms from primary tumor-to-metastasis seeding? Can we specifically target this process? The answers to these questions will inform the development of new therapeutic strategies against metastatic spreading, especially tertiary metastasis from bone to other visceral organs, as bone-only single-site metastasis is usually a confined disease and associated with better survival as compared to metastases in visceral organs [79].

Metastatic Bone Marrow Niches

Bone metastases occur most frequently in the axial skeletons, including the skull, the rib cage, and the spine. These sites are also where hematopoiesis mainly occurs [2]. Normal hematopoiesis is sustained by **hematopoietic stem cells** (HSCs), which reside mainly in specialized bone marrow niches (Box 2) [80,81]. HSC niches regulate the long-term quiescence, self-renewal, and differentiation of residing HSCs. DTCs may compete with HSCs for niche support [82], and therefore, may be regulated by the niche in a similar fashion. Moreover, DTCs utilize similar molecules as HSCs to interact with niche components, such as CXCL12–CXCR4, parathyroid hormone-related protein (PTHrP)-receptor, Jagged1-Notch, and heterotypic adherens junctions [62,67,83–85], suggesting that the knowledge of HSC niches may be applied to metastatic niches for tumor cells. In the following sections we will discuss the candidate niche cells and how they contribute to bone metastasis (Figure 3, Key Figure).



Trends in Cancer

Figure 2. Mechanism of Osteoblastic and Osteolytic Metastasis. Both osteoblastic and osteolytic metastases involve the interactions between tumor cells, osteoblasts, and osteoclasts. Tumor cells directly and indirectly alter the balance between RANK ligand (RANKL) and its antagonist osteoprotegerin (OPG), which has profound effects on bone homeostasis. In osteoblastic lesions, tumor cells secrete cytokines to promote osteoprogenitor cell recruitment and differentiation, as well as osteoblast proliferation. The activated osteoblasts may create a tumor favorable environment by producing bone matrix proteins and growth factors. Due to the coupling of osteoblast and osteoclast activities, osteoclasts are also stimulated in osteoblastic lesions. However, the overall bone resorption rate is lower than that of bone formation, possibly due to a relatively low ratio of RANKL to OPG in the environment. Thus, the net effect on bone is an abnormal increase in bone mass. By contrast, osteolytic tumor cells secrete osteolytic factors such as parathyroid hormone-related protein (PTHrP) and interleukin-11 (IL-11), which induce osteoblast production of RANKL, therefore promoting osteoclastogenesis. Osteolytic tumor cells can also directly activate osteoclasts through expressing RANKL, Jagged1, and metalloproteinases. Increased osteoclastic activity leads to bone destruction, and releases growth factors and calcium from the bone matrix, which in turn support the expansion of tumor cells. In multiple myeloma, tumor secretions can also inhibit the differentiation of osteoprogenitor cells and contribute to the reduced bone formation. Abbreviations: IGF, insulin-like growth factor; TGFβ, transforming growth factor beta proteins.

The endothelium is the first cellular barrier that circulating tumor cells may encounter in the bone marrow. Tumor cells must arrest in blood vessels, adhere to the endothelium, and extravasate into the parenchyma of bone marrow. *In vitro* cell adhesion experiments showed that bone-tropic tumor cells preferentially adhere to bone marrow endothelium as compared to endothelial linings from other organs, suggesting that the initial landing of tumor cells in bone is not a completely stochastic event [86]. By intravital imaging, the engraftment of fluorescently tagged tumor cells into bone marrow was found to occur at a specialized perisinusoidal region, which expresses E-selectin and CXCL12 [87]. Extravasation may not be a rate-limiting step due to the discontinuous and permeable nature of bone marrow endothelium [88]. After extravasation, the

Box 2. Hematopoietic Stem Cell Niches in Bone Marrow

The mapping of the HSC niche represents an area of ongoing active research. Two anatomically different HSC niches have been proposed based on their location in the bone, namely, the endosteal niche and the perivascular niche [80,81].

The endosteal niche (or osteoblastic niche) is immediately adjacent to the osteoblastic lining cells of the inner bone surface. Nilsson *et al*. [163] demonstrated that *ex vivo* labeled HSCs preferentially seed in the endosteal region after being transplanted into mice. Increase in the number and activity of osteoblasts through genetic modulation of PTH/PTHrP receptors or BMP signaling was reported to correlate with increased number of HSCs [164,165]. Later, Ang-1, mainly produced by osteoblastic cells, was demonstrated to activate Tie2 on HSCs and enhance the quiescence and survival of HSCs by promoting tight adhesion between HSCs and osteoblasts [166]. However, the direct effects of endosteal niches in regulating HSCs have been challenged later by several studies showing that manipulations of the adhesion molecules either on HSCs or osteoblasts do not affect HSC numbers [167–169].

One the other hand, in a landmark study, Kiel *et al*. [170] proposed that HSCs are localized to the perivascular niche. Subsequent studies suggested that these perivascular HSCs may reside next to sinusoidal blood vessels (the perisinusoidal niche) or arterioles (the periarteriolar niche) [171–176]. In both niches, endothelial cells support HSCs through direct cell–cell contact and paracrine factors, including E-selectin, Notch, CXCL12, and SCF [172–174,177,178]. Other perivascular stromal cells broadly defined as MSCs also play a role [171,172,179,180]. Specifically in the perisinusoidal niche, MSCs expressing various markers have been shown in mouse models to support HSC maintenance, including CXCL12-abundant reticular (CAR) cells, Nes–GFP dim, LepR–Cre+, or Prx-1–Cre+ cells [174]. Notably, some of these marker-defined cell populations are overlapping [181]. For example, CXCL12–GFP+ cells are also targeted by Prx-1–Cre and about 80% of LepR–Cre+ cells are also Nes–GFP+ [182]. In the periarteriolar niche, HSCs are regulated by Nes–GFP bright and NG2–Cre+ MSCs, and conditional depletion of NG2–Cre+ cells leads to the exhaustion of HSCs [175].

tumor cells may remain adjacent to endothelial cells, and be kept dormant by the perisinusoidal environment [89]. Ghajar *et al*. [90] demonstrated that the dormancy of perivascular mammary tumor cells is induced by TSP-1 secreted by endothelial cells. A recent study suggests that endothelial cells may transdifferentiate into osteoblasts in prostate cancer [91], adding further complexity to the role of endothelial cells in maintaining dormancy.

While residing in the perivascular niche, tumor cells may also be influenced by other perivascular stromal cells, specifically MSCs. The roles of MSCs in bone metastasis are debatable. MSCs have been demonstrated to facilitate tumor cells extravasation into bone marrow via secreting CXCL12 [92,93]. However, a subpopulation of MSCs express both endothelial and pericyte markers, and was shown to suppress the homing of cancer cells to bone [94]. While several studies showed that tumor cells may enter a quiescent state when co-cocultured with bone marrow-derived MSCs [95–99], the proliferation-promoting effects of MSCs were also reported [62,100–102]. The seemingly contradictory roles of MSCs in bone metastasis may reflect the phenotypic heterogeneity of this cell population, as discussed in the context of perivascular niches for HSCs (Box 2).

The central role of osteoblasts in bone metastasis has been extensively discussed. For instance, prostate cancer cells have been demonstrated to preferentially seed to the osteoblast-rich area in bone [103]. The increased secretion of growth factors by prostate tumor cells may promote recruitment, proliferation, and differentiation of osteoprogenitor cells, leading to osteoblastic metastasis [67]. Tumor-entrained pre-osteoblasts were shown to promote prostate cancer cells growth in a noncontact coculture system [104]. For breast cancer, our work demonstrated that osteogenic cells form a growth supportive niche for ERα+ breast cancer cells in the early stage of bone metastasis [62,66]. The interaction between osteogenic niches and residing tumor cells is mediated through adherens and gap junctions, which confer survival advantage to tumor cells via activation of mechanistic target of rapamycin (mTOR) signaling and the calcium influx from niche cells [62,66]. Osteoblasts also play a role in osteolytic bone

Key Figure

Bone Marrow Niches for Metastatic Tumor Cells

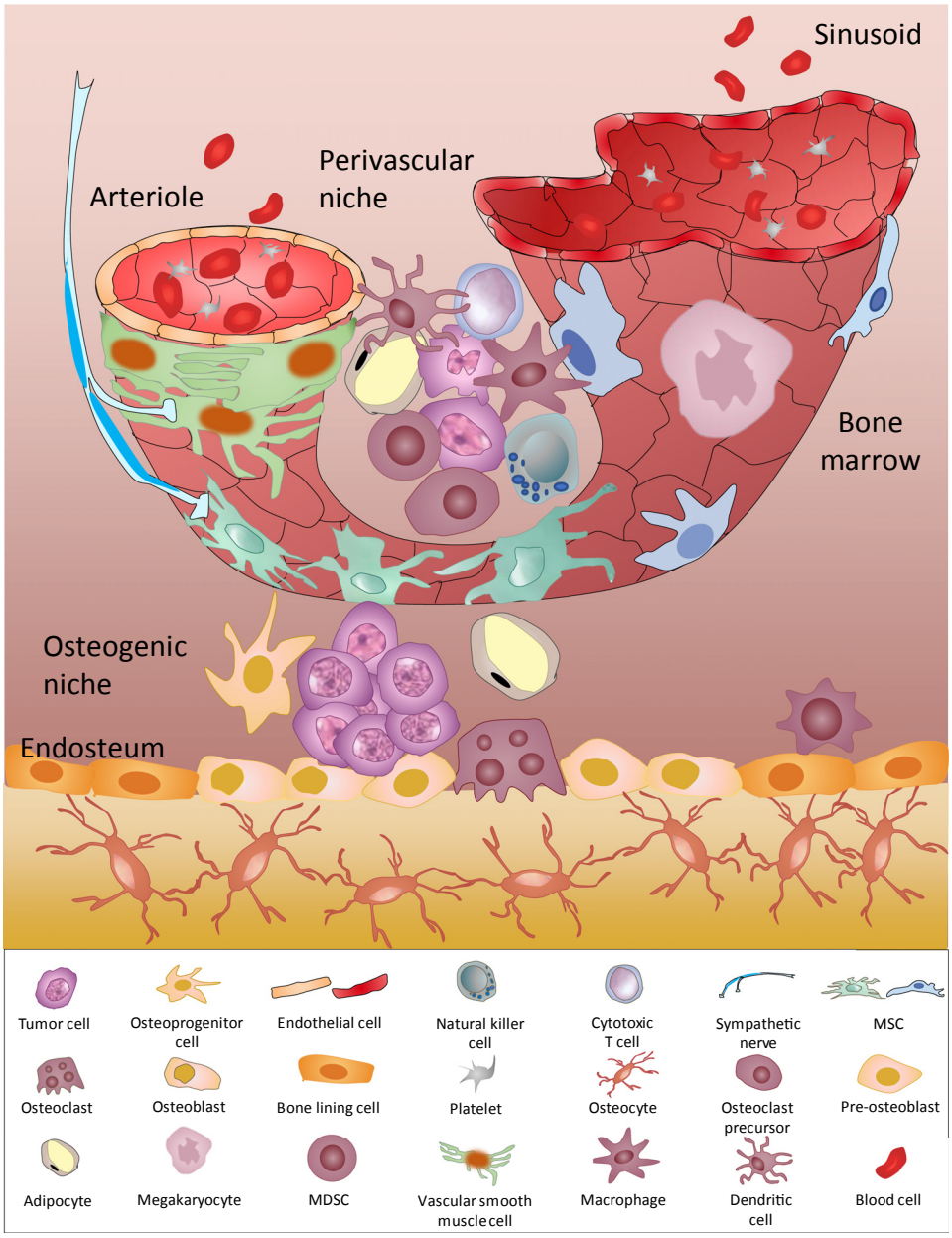


Figure 3. Generally, two different bone marrow niches may host tumor cells. Osteogenic niches (or endosteal niches) are adjacent to the endosteum, which are comprised mainly of osteoblastic cells. Our work suggested that osteogenic cells establish physical connection with residing tumor cells through heterotypic adherens junctions and gap junctions. This interaction activates the mechanistic target of rapamycin (mTOR) pathway in cancer cells and triggers calcium influx from niche cells, which promotes cancer cell survival and proliferation. By contrast, the perivascular niche is proposed to

(Figure legend continued on the bottom of the next page.)

lesions. Bone-residing tumor cells secrete factors such as PTHrP and IL-11, which induce RANK ligand (RANKL) secretion and reduce osteoprotegerin (OPG) production in osteoblastic cells [8–10]. These molecular changes subsequently stimulate osteoclast development, drive the osteolytic vicious cycle, and promote bone resorption and tumor growth [10]. Interestingly, despite these aforementioned studies, there is also evidence suggesting an opposite role of osteoblasts in promoting dormancy, rather than progression. By intravital imaging, dormant myeloma cells were found to directly contact the endosteal surface, which suggests that osteoblastic niches may be dormant niches in the context of myeloma [105]. Similarly, the secretome of osteoblastic cells induced quiescence of DTCs in prostate cancer models [106,107]. These inconsistent conclusions may reflect the complicated natures of the seed–soil interaction. On the one hand, cancer cells that contact osteoblasts using different mechanisms (e.g., adherens junctions vs. focal adhesion) may activate different intrinsic signals. On the other hand, osteogenic cells in variable functional and differentiation statuses may exert different influence on the microenvironment. Thus, the precise effects of microenvironment niche need to be investigated *in vivo* at a single-cell resolution and in models that recapitulate relevant disease features (e.g., the ER status of breast cancer) in the clinic.

Osteoclasts are a main force in remodeling the bone environment. In osteolytic lesions, osteoclasts are highly stimulated by cancer cells and osteoblasts to resorb the bone matrix. This releases deposited growth factors in bone, which in turn support the growth of tumor cells and osteoblasts. Therefore, a positive feedback is established, leading to osteolytic vicious cycle [10]. Interestingly, the activity of osteoclasts is also increased in osteoblastic metastasis, suggesting the tight functional connection between osteoblasts and osteoclasts [108]. In multiple myeloma, osteoclasts were reported to remodel the endosteal niches and thereby activate the residing dormant tumor cells [105]. Osteoclast precursors have been shown to share similar markers with myeloid-derived suppressor cells (MDSCs), and exert T cell suppressive activity in a murine model of rheumatoid arthritis [109,110]. An *et al*. [111] demonstrated that osteoclasts attenuate T cell-mediated cytotoxicity via upregulation of a series of immune checkpoint molecules in myeloma. Thus, osteoclasts may play multiple roles other than bone remodeling in the context of bone metastasis.

Immune cells are another major cell population in the bone marrow. Compared to the extensively studied primary tumors, much less is known about the immune landscape in bone metastasis. Since the bone marrow is composed of a wide variety of immune cells [112], both antitumor and protumor immune cells may participate in or influence the bone metastatic niche. Active CD4, CD8, natural killer T cells, and natural killer (NK) cells may all contribute to the immune surveillance and eradication of bone metastasis [63,113–115]. Depletion of T cells and NK cells accelerated bone metastasis in a murine breast cancer model [63]. By contrast, active CD4 T cells may also have prometastasis roles. They were shown to promote osteoclastogenesis and induce premetastatic osteolytic lesions, which may facilitate the colonization of myeloma and breast cancer cells in bone [116,117]. In addition, regulatory T cells can suppress

be a dormancy permissive niche. TSP1 from endothelial cells maintains tumor cells in a dormant status. Other stromal cells in the perivascular niche are highly heterogenous, including mesenchymal stromal/stem cells (MSCs) expressing NG2+, Nes–GFP+, LepR+, or C–X–C motif chemokine ligand 12 abundant reticular cells. As such, the perivascular niches may exert complex effects on residing tumor cells. Given that the endosteum is also highly vascularized, it is plausible that osteogenic niches and perivascular niches may spatially overlap in the endosteal region. In addition, MSCs can undergo osteogenic differentiation to generate osteoblastic lineage *in vivo*. Other cells types found in the bone marrow, including lymphocytes, myeloid-derived suppressor cells (MDSCs), macrophages, adipocytes, osteoclasts, megakaryocytes, and sympathetic nerves, can directly and indirectly participate in these two niches to orchestrate the progression of bone metastasis.

osteoclast formation as well as cytotoxic T cell function, and therefore, contribute to immune evasion and increased bone deposition in prostate cancer bone metastasis [118,119].

Myeloid cells mostly assume immunosuppressive roles in the cancer setting. MDSCs are a heterogenous group of immunosuppressive cells. They include immature monocytic cells, neutrophil, and dendritic cells [112,120], and constitute up to 20–30% of all bone marrow stromal cells [112]. The effects of MDSCs on lymphocyte effector were mainly investigated in primary tumors. Their involvement in the bone marrow niche was also observed in multiple myeloma [121]. Besides the immunosuppressive function, MDSCs may be able to differentiate into osteoclasts, which may subsequently promote bone loss in bone metastasis [122,123]. Plasmacytoid dendritic cells were found to be increased with bone metastasis in a breast cancer model, and depletion of this population could ameliorate bone loss and suppress tumor growth in bone [124]. In addition to MDSCs, macrophages represent another major myeloid cell population. In prostate cancer patients as well as mouse models, $CD206^+$ M2-like macrophages were observed to be enriched in the growing metastatic lesions in bone [125,126]. Conditional genetic depletion of tissue-resident macrophages reduced tumor burden in bone in mice carrying prostate tumors [126]. In summary, the diversity of immune niches is just beginning to be appreciated, and accordingly, systemic investigation is required to elucidate the link between immune system and bone homeostasis as well as bone metastasis.

Other cell types have also been implicated in regulating bone metastasis. For instance, the activation of sympathetic nerve is accompanied by increased infiltration of M2 macrophages in primary tumors and thereby promote metastasis at distant organs including bones [127]. In addition, the sympathetic nervous system has been suggested to stimulate bone marrow stromal cells and promote breast cancer bone metastasis in mouse. Increasing evidence also shows that bone marrow adipocytes can attract and interact with metastatic tumor cells, and provide an alternative source of growth factors and energy need supporting metastatic growth [128]. When cocultured with human bone tissues *ex vivo*, breast cancer cells were observed to preferentially colonize into the adipose tissue compartment [129]. By contrast, platelets were demonstrated to be critical in the preparation of premetastatic niches, but limited reports are available concerning bone metastasis [36]. Targeting platelet aggregation by inhibiting activated integrin $\alpha_{IIb}\beta_3$ significantly prevents the onset and later progression of bone metastasis in mouse models [12,130]. Platelets not only protect circulating tumor cells from immune clearance, but also promote the osteolytic vicious cycle in bone [131]. The lysophosphatidic acid derived from platelets was shown to promote skeletal tumor growth and bone resorption by stimulating pro-osteoclast cytokines [130]. By contrast, their precursor cells, megakaryocytes, have been reported to correlate with increased bone mass and to negatively associate with skeletal metastasis in a murine prostate metastasis model [132]. In a recent study, increased number of megakaryocytes was found to be associated with the metastatic growth of breast cancer cells in bone in both mouse models and human specimens [133]. However, mice with deficient megakaryocytes developed more aggressive bone metastasis as compared to the wild-type hosts, suggesting that the increase of megakaryocytes in bone marrow could be a protective mechanism against bone metastasis in breast cancer [133].

Current Limitations

The interaction between tumor cells and bone cells is crucial for the successful colonization of tumor cells in bone. Bone marrow niches not only provide a 'sanctuary' for tumor cells to escape adjuvant therapies but also serve as a 'cradle' of evil seeds to fuel local and distant relapse. A lot remains unknown (see Outstanding Questions) about the bone marrow niches for skeletal metastasis due to several challenges. First, the current preclinical models for bone

Box 3. The Complexity of Hematopoietic Stem Cell Niches

The complexity of HSC niches is under active investigation.

Firstly, more bone marrow stromal cells are recently implicated to either directly or indirectly regulate HSCs, including osteoclasts, macrophages, adipocytes, megakaryocytes, and sympathetic nerves [183–187].

Secondly, recent studies have revealed the heterogenous nature of bone marrow niches. For instance, HSCs have different reactive oxygen species levels at arteriolar and sinusoidal sites due to the different vascular permeability, which in turn regulate their migration, differentiation, and survival [188]. Moreover, endothelial cells at perisinusoidal and periarteriolar niches differ in their expression of surface markers, their secretion of niche factors as well as their functional contribution to HSC niches [189]. Interestingly, the same cytokine factors also have different contributions to HSC maintenances at different perivascular sites [190]. For example, selective ablation of CXCL12 from arteriolar NG2–Cre+ cells, but not from sinusoidal LepR–Cre+ cells, reduces HSC numbers [190].

Thirdly, the niches and residing HSCs are not static. Endothelial cells are shown to remodel to surround HSCs after their arrival at the perivascular niches [191]. MSCs, which are close to the endothelial pocket, were shown to orientate HSC division [191].

Lastly, the endosteal niches and perivascular niches may overlap to some extent. Perivascular MSCs are known to be precursors of osteoblastic cells, and importantly, the endosteal surface is also highly vascularized, suggesting that the endosteal niche and perivascular niche may be a common niche. This can be supported by a recent study showing that the endosteal but not central bone marrow vasculatures were degraded and correlated with the loss of HSCs in acute myeloid leukemia model [192]. Taken together, all of the aforementioned studies suggest the dynamic and complex nature of HSC niches.

Outstanding Questions

What are the mechanisms underlying the homing of DTCs into a specific niche? Is it a stochastic process or a selective process for tumor cells with specific traits?

Are there different niches for different metastatic seeds? Do different niches carry out different functions during bone metastasis? How do niche components coevolve with the residing tumor cells?

How will systemic changes, such as obesity, aging, bone-modifying agents (e.g., bisphosphonates), and cancer treatments (e.g., endocrine therapies and chemotherapies), influence the niches? Can these conditions lead to redistribution of tumor cells among different niches, and consequently alter the kinetics of bone colonization?

Can cancer cells migrate from one niche to another and thereby change their fates? If so, what are the underlying mechanisms?

Do bone marrow niches educate residing tumor cells and promote further dissemination?

What therapeutic opportunities can we get from a better understanding of the interaction between tumor cells and the bone marrow niches in bone metastasis?

metastasis are far from ideal. Few models can faithfully recapitulate the natural progress of bone metastasis, particularly the extended latency observed in patients, which poses a major limitation and hinders the translational application of preclinical research. In addition, more clinically relevant models, like syngeneic mouse models with hormone-responsive tumors, are required for future study of immune regulation of bone metastasis. Besides better models, new approaches are required to capture the interaction between tumor cells and host cells at the single-cell level. Although many cell types may contribute to bone colonization, we need to identify the key ones that directly promote progression and are therapeutically targetable. That requires a much more refined and precise map of where the different niches are located, and their spatial relationship with bone metastases at different stages. The studies on HSCs provide us with a complex map of bone marrow niches (Box 3). Moreover, the map of bone marrow niches is dynamic. The bone constantly undergoes turnover, which can be influenced by many processes including menopause, aging, and drug treatments. It is likely that the metastatic niches evolve during these events, which in turn alter the fate of resident tumor cells. For instance, the decrease in estrogen level due to either menopause or endocrine therapies could significantly enhance osteoclast-mediated bone resorption by increasing the expression of *RANKL* while reducing the expression of *OPG* [134]. Src inhibitor dasatinib was shown to accelerate the differentiation of MSCs into osteoblasts but inhibit osteoclast activity [135,136]. Chemotherapy agents induce Jagged1 expression in osteoblastic lineage through the reactive oxygen species pathway, and subsequently promote the seeding of cancer cells to bone and their resistance to chemotherapy [84]. Finally, a combination of single-cell sequencing, lineage tracing, and cutting-edge microscopy may help us further refine this map and capture this complex and dynamic interaction between cancer cells and various niches.

Concluding Remarks

Bone metastasis is currently still incurable. Targeting the tumor–niche interaction and the niche components represents a promising direction of future therapeutic strategies to eliminate minimal residue disease and to prevent overt metastasis. For instance, the therapeutic antibody against Jagged1 on tumor cells and osteoblastic niches was shown recently to significantly reduce bone metastasis and overcome niche-induced chemotherapeutic resistance [84]. Our

recent work suggests that a short interlude of treatment with everolimus plus arsenic trioxide could diminish the survival advantage of DTCs conferred by osteogenic niches, and significantly prevent the long-latency bone metastasis in mice [66]. Pharmacological modulation of the metastatic 'soil' toward a normalized bone environment can also ameliorate bone metastasis. A good example here is the application of bisphosphonates in treating bone metastasis. Although zoledronic acid plus current standard adjuvant care failed to show additional overall benefit in patients with early breast cancer, it reduced the frequency of bone metastasis and improved disease-free survival in postmenopausal patients or in patients with low estrogen level [137–142]. In addition to potential antitumor effects, their well-known function is to inhibit the increased bone resorptive activity of osteoclasts [143]. Importantly, bisphosphonates can also repolarize M2-like tumor-associated macrophages to M1-like antitumor phenotype and activate T cells [144,145]. Given the tight link of bone and immune system, it is rational to test the combination of immune checkpoint therapy with current therapies in treating bone metastasis.

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Bone Tropism in Cancer Metastases

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Bone is a frequent site of metastases in many cancers. Both bone properties and the tumor-intrinsic traits are associated with the metastatic propensity to bone (i.e., the bone tropism). Whereas an increasing body of mechanistic studies expanded our understanding on bone tropism, they also revealed complexity across the bone lesions originated from different cancer types. In this review, we will discuss the physical, chemical, and biological properties of bone microenvironment, identify potential players in every stage of bone metastases, and introduce some of the known mechanisms regulating the bone colonization. Our objectives are to integrate the knowledge established in different biological contexts and highlight the determinants of bone tropism.

Metastasis is the direct cause of more than 90% of cancer-related deaths (Seyfried and Huysentruyt 2013). In many cases, tumors relapse in distant organs years or even decades after resection of the primary tumor (Obenauf and Massagué 2015). Bone is one of the most frequent sites of metastases. In the clinic, bone metastasis remains an incurable disease, which is responsible for morbidity in most advanced breast and prostate cancers, and characterized by severe pain, impaired mobility, pathologic fractures, numbness, paralysis, anemia, and hypercalcemia leading to coma and death (Coleman 2006). Bone tropism is the propensity and capacity of tumor cells to spread and eventually become clinically evident in bone (Fatatis 2011). Understanding the bone tropism will help us to specify the behavior and mechanism of bone metastases from that of the primary tumor and other metastases, and pave the way for effective clinical applications.

BONE TROPISM IN DIFFERENT CANCER TYPES

The incidence of bone metastases and median-survival of bone metastasis patients vary greatly among different cancer types, which is summarized in Table 1 (Selvaggi and Scagliotti 2005; Macedo et al. 2017). Bone metastases also vary in different cancer types by specific mechanisms to disrupt bone homeostasis. The osteolytic metastases, characterized by destruction of bone structure and loss of bone mass, are detected mostly in breast cancer, lung cancer, multiple myeloma, melanoma, and renal cell carcinoma. In contrast, the osteoblastic metastases, featured by excessive bone formation, are found predom-

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Table 1. Incidence of bone metastases in cancer

Primary cancer type	Relative incidence in bone	Median survival from diagnosis
Breast	65%–75%	19–25 mo
Prostate	65%–75%	12–53 mo
Lung	30%–40%	6 mo
Thyroid	40%–60%	48 mo
Bladder	40%	6–9 mo
Renal	20%–25%	12 mo
Melanoma	14%–45%	6 mo

Data in the table based on Suva et al. (2011) and Macedo et al. (2017).

inantly in prostate cancer, as well as a minority of breast and lung cancers. In fact, lesions exhibiting both osteolytic and osteoblastic activities are often observed in patients with advanced breast cancer and prostate cancer (Suva et al. 2011). Thus, even within the same type of cancer, bone metastasis features may vary widely.

Breast Cancer

In the clinic, metastasis organ tropism and latency vary significantly across different molecular subtypes of breast cancer. For instance, luminal A/B subtypes show a lower recurrence rate (27.8%–42.9%) than the HER-2+ tumors (51.4%) as well as a longer metastasis-free survival compared to HER2 and basal subtypes (1.6–2.2 yr vs. 1.3 and 0.7 yr), indicating that the luminal subtype is less aggressive in general. Nevertheless, luminal tumors exhibit a higher incidence of bone metastasis (62.1% and 64.5%) than the HER2+ subtype (47.7%) and basal subtype (32.2%) (McGuire et al. 2015). The luminal subtype is characterized by the positive expression of estrogen receptors (Wiechmann et al. 2009). Consistently, the overall rate of bone metastasis is much higher in patients with ER+ tumors than those with ER− tumors (71% vs. 47%) (Smid et al. 2008; Zhang et al. 2009; Kennecke et al. 2010). In spite of the lower bone relapse rate, ER− tumors often develop bone relapse within a shorter time than ER+ tumors (5 yr vs. 10 yr) (Kennecke et al. 2010), indicating that the mechanisms of metastasis may differ between different subtypes. It remains unclear whether the difference in bone tropism is due to a stronger advantage conferred on the luminal cancer cells by the bone microenvironment, or a consequence indirectly caused by incompetency of luminal cancer cells in quickly colonizing visceral organs. In other words, the basal subtype may have equal or even stronger capacity to form bone metastasis, but only lacks sufficient time to manifest—as patients usually succumb to other metastases first. Further studies are needed to address these different possibilities.

Prostate Cancer

Prostate cancer exhibits a strong preference for bone metastasis. In autopsies of patients with advanced prostate cancer patients, metastases were much more frequently found in bones than in other organs (Bubendorf et al. 2000). Prostate cancer skeletal metastases are most often osteoblastic (characterized by increased mineral density), which are the opposite to osteolytic metastases predominantly occurring in breast cancer (Macedo et al. 2017). Androgen-deprivation therapy (ADT), the elimination of testosterone by medical or surgical castration, is the major treatment of prostate cancer (Andriole 2009). However, many patients will eventually develop the castration-resistance within 18–24 mo after ADT. As the disease progressed, more than 90% of patients with metastatic castration-resistant prostate cancer (mCRPC) will develop bone metastases (Frieling et al. 2015). The potential mechanistic links between resistance to ADT and bone-tropic metastases remain poorly studied. Preclinical data derived from xenograft models suggested that the loss of prostate-specific antigen (PSA) and androgen receptor (AR) might be associated with the osteolytic phenotype in prostate cancer bone metastases (Dai et al. 2016), which is yet to be clinically validated.

Lung Cancer

Lung cancer is the third most common form of cancer to spread to bone, after breast and prostate cancer (D'antonio et al. 2014). The median

survival of a patient with lung cancer bone metastasis is 6 mo, which is the shortest among all cancer types metastasizing to bone (Vicent et al. 2015). Notably, as one of the major indicator of lung cancer, the epidermal growth factor receptor (EGFR) status strongly correlates with the occurrence and pathologies of bone metastases (Bethune et al. 2010). In non-small-cell lung cancer (NSCLC), EGFR+ patients display a strong predisposition to bone metastases, which justifies prospective bone metastasis screening on EGFR+ patients (Kuijpers et al. 2018). Moreover, although NSCLC bone metastases are osteolytic in general, osteoblastic lesion is more commonly found in patients with mutant EGFR lung adenocarcinomas, especially after TKI therapy (Ansén et al. 2010; Pluquet et al. 2010). These observations indicate functional connections between oncogenic EGFR activities and bone tropism of metastasis.

The diversity and complexity of bone metastases in the clinic pose a challenge to basic research due to limited choices of cell or animal models. This limitation may underlie observed incongruence among published results and the discrepancy between clinical and basic research (Prinz et al. 2011). Therefore, cautions should be used to interpret the findings in the context of specific cancer types/subtypes and to draw general conclusions about all bone metastases.

DETERMINANTS OF BONE TROPISM

Based on the "seed and soil" hypothesis made by Stephen Paget in 1889, the determinants of metastasis tropism include tumor-intrinsic traits (the "seed") and the characteristics of the host organs (the "soil") (Zhang et al. 2019). One hundred and thirty years after the hypothesis was proposed, a growing body of evidence indicates that the interactions between cancer cells and the distant organs is more than natural selection for the fittest. Rather, to successfully colonize, disseminated tumor cells (DTCs) must adapt to the new microenvironment, which involves a series of dynamic crosstalk that may lead to reprogramming of both "seed" and "soil." In the context of bone, the metastatic colonization process is arguably more complicated, due to the prolonged temporal course and the uniqueness of the bone environment.

PHYSIOCHEMICAL PROPERTIES OF BONE: THE SOIL

Bone is a mineralized tissue featured by intense vascularization, low oxygen level, high local calcium concentration, and acidosis (Johnson and Suva 2018). All these characteristics make the skeleton a unique and perhaps challenging environment for DTCs to colonize, survive, and proliferate.

Sinusoid Structure

Being arrested in the capillary bed is the first step of successful bone colonization (Mundy 2002). Due to the discontinuous and fenestrated endothelium of sinusoids, extravasation is not expected to be a rate-limiting step, making bone one of the most accessible organs to the circulating tumor cells (Nguyen et al. 2009; Esposito et al. 2018). Perhaps because of this, although breast cancer cells are capable to colonize multiple distal organs, bone is often the first site of metastasis (Suva et al. 2009). Even for the cancers that rarely develop overt bone metastases such as gastrointestinal cancer, DTCs can yet be found in the bone marrow (Hiraiwa et al. 2008).

Vasculature and Niches

Bone is a highly vascularized tissue. Accumulating evidence shows that blood vessels in the bone may provide an initial harbor for tumor cells and contribute to the subsequent processes of bone metastasis (Raymaekers et al. 2015). The perivascular niche, mainly formed by endothelial cells and perivascular stromal cells, is essential for dormancy maintenance in cancer cells (Ghajar et al. 2013; Price et al. 2016). In contrast, osteogenic niche (or endosteal niche), which is derived from mesenchymal stem cells and comprises osteoblasts and progenitor cells, has been shown to be a niche fueling proliferation (Wang et al. 2015).

How cancer cells transit between the endothelial niche and osteogenic niche, and how such a transition alters the fate of tumor cells remain to be elucidated. Some studies indicated that migration of cancer cells from the perivascular niche to the osteoblastic niche is mediated by various expression of cytokines (CXCL12, RANKL, PTHrP) in different niches (Esposito et al. 2018). Alternatively, the niche components may undergo trans-differentiation to switch niche functions. In prostate bone metastasis, the endothelial cells could be converted to osteoblasts via BMP4 secreted by cancer cells (Lin et al. 2017). Therefore, the seemingly different niches may actually share the same cell-of-origin. Consequently, cancer cell fate may be altered without spatial movement.

Of note, the bone metastatic niches may be the hematopoietic stem cell (HSC) niche. The endosteal niche is found important for both DTCs and HSCs by physical adhesion and attachment (Adams et al. 2007; Wang et al. 2015). One study on bone metastasis in prostate cancer showed that DTCs compete against HSC for the same niches in the bone marrow (Shiozawa et al. 2011). Mechanistically, DTCs and HSCs may similarly use the CXCL12/CXCR4 signaling to home and survive (Broxmeyer 2008; Teicher and Fricker 2010; Shiozawa et al. 2011). Considering these commonalities, further studies on HSC niche may generate valuable insights into bone metastasis, and vice versa.

Low Oxygen Level

Despite being a highly vascularized tissue, bone is a particularly hypoxic environment. Oxygen tension in normal tissues falls between 2% and 9% (14–65 mmHg); in the periosteum and cortical bone, oxygen levels range from 4.2% to 7% (30–50 mmHg). In the bone marrow where bone metastases initiate and develop, the oxygen level is usually lower than 2% (pO2: Trabeculum: 0%–2%; Endosteum: ~1.8%; Sinusoidal regions: ~1.3%) (Johnson et al. 2017). Previous studies demonstrated that under low oxygen condition, cancer cell secretome may be altered to facilitate metastatic colonization and osteoclast differentiation (Gilkes 2016). In breast cancer, hypoxia (HIF-1 pathway) alone or together with TGF-β signaling promote the development of osteolytic bone metastases, which could be pharmacologically inhibited by anti-HIF1 treatment (Hiraga et al. 2007; Dunn et al. 2009). Additionally, the HIF-1 pathway also confers chemotherapy resistance on triple-negative (TN) breast cancers, supporting the rationale to combine HIF inhibitors with conventional cytotoxic chemotherapies (Samanta et al. 2014). Notably, most evidence for bone metastasis-specific impact of hypoxia was derived from fast-growing cells (e.g., human MDA-MB-231 cell lines and murine 4T1 cell lines), which largely bypassed the indolent growth period of bone metastasis. Studies using more latent tumor models (e.g., ER+ breast cancer and osteoblastic prostate cancer) are of urgent need to deepen our understanding of hypoxia in the context of bone metastases (Johnson et al. 2017).

Calcium

Bone is a mineralized tissue. Compared to the normal physiological calcium level (~1.1–1.3 mmol/L), the local calcium level in bone varies widely from 2 mmol/L (nonresorbing bone) to as high as 8–40 mmol/L (resorbing bone) (Liao et al. 2006). Therefore, the high local calcium level might render cancer cell addictive to calcium signaling. This is exemplified by the prevalent up-regulation of calcium-sensing receptor (CaSR) in bone metastases from breast cancer, prostate cancer and renal cell carcinoma (Sanders et al. 2000; Liao et al. 2006; Joeckel et al. 2014; Frees et al. 2018). The extracellular calcium is not the only accessible resource to cancer cells. During the early bone colonization of breast cancer, osteogenic cells and cancer cells establish a physical connection through gap junctions which directly transfer calcium influx from the osteoblasts to the cancer cells. The calcium influx can promote the calcium signaling like NFAT and MEF2 in cancer cells and hence leads to progression of bone metastasis (Wang et al. 2018). In addition, in advanced bone metastases with skeletal muscle weakness, increased bone destruction and associated elevations in TGF-β activity can oxidize RyR1 and therefore, lead to

Ca^{2+} leakiness from the muscle (Waning et al. 2015), which serves as another possible calcium reservoir for cancer cells.

MECHANISMS OF BONE METASTASIS: HOW THE SEEDS FIT IN THE SOIL

The journey of metastases is a stepwise cascade that includes (1) local invasion, (2) intravasation, (3) survival in circulation, (4) arrest in a distant organ, (5) extravasation, (6) micrometastasis, and (7) macrometastases (Obenauf and Massagué 2015). The preseeding steps 1–3 are likely to be shared by all distant metastases. In this section, we will focus on the postseeding steps by reviewing the unique biology of bone metastases.

Seed Preselection and Premetastatic Niche

Bone metastasis tropism may emerge even before cancer cells leave primary tumors. This may be driven by selective pressure exerted by the microenvironment of bone-like characteristics. A role of the breast tumor stroma in preselecting bone-tropism cancer cells was revealed in TN breast cancer. Cancer-associated fibroblasts (CAFs) in TN breast tumors produce CXCL12 (also known as SDF-1) and IGF1, two of the cytokines abundantly found in the bone microenvironment. Chronical exposure to these cytokines led to the enrichment of cancer cells with elevated c-Src activity (Zhang et al. 2013). Should these metastatic seeds arrive in the bone marrow, the c-Src activity would confer survival advantages through CXCL12–CXCR4 and IGF1–IGF1R pathway. Therefore, metastatic seeds with bone tropism are preselected in primary tumors due to the resemblance of the microenvironment in certain tumors to bone marrow.

Bone metastasis tropism may also emerge by preparing "soil" before metastatic seeds arrive. This concept is referred to as "premetastatic niche": the primary tumors secrete factors and extracellular vesicles into circulation, which may alter the microenvironment to facilitate metastatic seeding in distant organs (Peinado et al. 2017). A recent study showed that exosomes from lung-, liver-, and brain-tropic cancer cells preferentially fuse with resident cells in their targeted organs, thereby establish a favorable microenvironment for distant metastasis via expression of specific integrins (Hoshino et al. 2015). The exosome-induced bone tropism was not elucidated in this study, which needs to be further explored in the future. Other reports demonstrate that integrins expressed by cancer cells, such as α2β1, α5β1, and αvβ3, mediate bone metastasis (Weilbaecher et al. 2011). Whether exosomes carrying these integrins create premetastatic niche remains to be tested. In addition to integrins, exosomal miRNA may also play roles in the premetastatic niche of bone metastases. For instance, miR-940 can be delivered by exosomes from primary prostate cancer to the bones, and induce extensive osteoblastic lesions by facilitating osteogenic differentiation (Hashimoto et al. 2018). Similarly, exosomal miR-141-3p from MDA PCa 2b cells promoted osteoblast activity, making it more permissive for bone metastases of prostate cancer (Ye et al. 2017).

The lysyl oxidase (LOX) may promote the premetastatic niche in bone. Through circulation, LOX secreted by primary breast tumors facilitates osteolytic lesion formation in the skeleton of ER− patients (Cox et al. 2015). Additionally, the LOX-induced osteoclastogenesis is driven by the nuclear translocation of NFATc1, which is independent of the conventional RANKL signaling. Another study reported that colorectal cancer-derived LOX also promotes osteoclast differentiation albeit via a RANKL-dependent mechanism (Reynaud et al. 2017).

In a more complicated fashion, lung cancer cells form a feedback loop with distant bone cells, to drive tumor progression. Preclinical and clinical studies reveal that lung cancers increase bone stromal activity by secreting soluble receptor for advanced glycation end products (sRAGE) to the bone. Consequently, osteocalcin-expressing (Ocn+) osteoblastic cells are activated, which facilitate the outgrowth of lung cancer by remotely supplying a distinct subset of tumor-infiltrating $SiglecF^{high}$ neutrophils (Engblom et al. 2017). In future study, it will be interesting to validate whether the increased Ocn+ osteoblastic cells can represent a premetastatic niche of secondary bone metastasis. **Q2**

While secretome-induced premetastatic niche is intriguing, it should be noted that that evidence based on mouse models should be interpreted with caution. In mice, the (primary) tumor/body ratio could reach as high as 1:20 or even 1:10 by weight. In contrast, in human patients, the primary tumor (e.g., breast cancer) is typically <5 cm in diameter (Narod 2012), which should not exceed 1/1000 of the total body weight. Therefore, experiments based on adoptive transfer of exosomes and other secreted molecules should be cautious to avoid supraphysiological concentration.

Dormancy

Metastases relapse years after diagnosis or resection of primary tumors. In prostate cancer, the metastatic cells showed a strong bone tropism, and may stay asymptomatic for over years (Sturge et al. 2011). In breast cancer, despite that cancer cells may disseminate to bone at a very early stage, a significant proportion of patients do not manifest overt bone metastases until years later (Chen et al. 2017). Therefore, dormancy represents one of the most important characteristics of metastatic progression in the bone.

There are two different types of dormancy genes. The first type genes may inhibit proliferation but are essential for retention and survival of DTCs specifically in the bone. For example, E-selectin and CXCL12 in the perivascular environment maintain disseminated breast cancer cells in a dormant state (Price et al. 2016). E-selectin inhibition effectively blocks the homing of cancer cells, whereas the inhibition of CXCL12/CXCR4 interaction repels the dormant micrometastases into circulation. The significance of E-selectin and CXCL12/CXCR4 signaling for bone metastases is further supported by other studies (Müller et al. 2001; Kang et al. 2003; Stübke et al. 2012), highlighting the notion that dormancy needs to be coupled with long-term survival and bone retention for later outgrowth to be possible.

The second type of dormancy-related genes may be merely growth inhibitory. Ghajar et al. found that TSP-1 expression around microvessel stalks induces sustained quiescence of breast cancer cells in the perivascular niche. Interestingly, the suppression was relieved when new vessels sprout, which stimulates outgrowth of micrometastases (Ghajar et al. 2013). This type of dormancy genes may inversely correlate with clinical recurrences. Examples include MSK1 (Gawrzak et al. 2018), LIFT (Johnson et al. 2016), BMP7 (Buijs et al. 2012; Kobayashi et al. 2012), and KAI1/CD82 (Bandyopadhyay et al. 2006). All these genes are expressed by cancer cells, and their suppression promotes bone metastases. It has become evident that the dormancy mechanism may be diverse and vary in different biological contexts. While the list of dormancy genes may continue to grow, provocative questions remains: how the expression of this gene is maintained or evolutionally selected throughout the prolonged dormancy if they do not confer a growth advantage on cancer cells, and how the expression is finally suppressed to terminate dormancy and resume metastatic outgrowth.

Early Colonization after Dormancy

The mere presence of DTCs in bone marrow does not necessarily lead to successful colonization. In order to survive and outgrow in bone, cancer cells need to adapt to the initially nonpermissive environment and hijack bone-specific cellular and molecular mechanisms to gain access to growth factors and cytokines. For example, CXCR4 is one of the most up-regulated genes in bone metastasis of breast cancer, and this is probably due to strong selective or adaptive pressure exerted by bone niche cells that express abundant CXCL12/SDF-1, the ligand of CXCR4. This interaction further elevates the c-Src signaling, which is strongly associated with long-term survival, the exit of dormancy, and therapeutic resistance (Myoui et al. 2003; Zhang et al. 2009; Chiu et al. 2017).

Cancer stemness and the epithelial-to-mesenchymal transition (EMT) are widely recognized to be important for distant metastasis (Kang and Pantel 2013). Specifically for bone metastasis, EMT has also been subjected to the intensive investigation (Gao et al. 2014). In pros-

tate cancer, a loss of PTEN is accompanied with RAS/MAPK signaling activation and induces EMT, leading to macrometastasis in bone with 100% penetrance (Mulholland et al. 2012). In breast cancer, DTCs found in the bone marrow aspirates are enriched with CD44+/CD24−/low cells (Balic et al. 2006). The CD44+/CD24− subpopulation is also more mesenchymal and stem-like (Mani et al. 2008), suggesting that EMT may be essential for early dissemination and homing to the bone. Another study demonstrated that expression of DKK1 endow mesenchymal-like DTCs capacity to evade immune surveillance by inhibiting Wnt signaling in local immune cells (Malladi et al. 2016), supporting that EMT may play roles in the long-term survival of DTCs before colonization starts.

After EMT-mediated dissemination and homing are accomplished, the ultimate colonization of the distant metastasis requires the restoration of epithelial status (namely, mesenchymal-to-epithelial transition [MET]) (van Denderen and Thompson 2013; Esposito et al. 2019). The epithelial trait is selected in colonization maybe because it allows cancer cells to adhere to other cells or extracellular matrix, and gain momentum of proliferation. Multiple proteins and pathways may mediate the cell–cell direction and alter the fates of DTCs, including VCAM-1/integrin (α4β1) (Lu et al. 2011), integrin (α9β1)/Tenascin C (San Martin et al. 2017), Jagged 1/Notch (Sethi et al. 2011), E-cadherin/N-cadherin (Wang et al. 2015), OB-cadherin (Chu et al. 2008), Connexin 43 (Wang et al. 2018), and Glg1/E-selectin ~~(Fig. 1;~~ Esposito et al. 2019). Thus, the static "epithelial" or "mesenchymal" state may not be the key determinant of metastasis capacity. Rather, the "plasticity" to swing between these states may be the essential property (Li and Kang 2016; Pastushenko et al. 2018). Indeed, cells expressing hybrid mesenchymal (e.g., vimentin) and epithelial (e.g., E-cadherin) markers were shown to be therapeutic resistant and exhibit enriched cancer stemness (Creighton et al. 2009). The dependency of cell–cell adhesion may be particularly strong in the bone because of tissue rigidity and stiffness, which make anchor-dependent growth a rate-limiting process (Johnson and Suva 2018).

In different cancer types/subtypes, the exit of dormancy and outgrowth of macrometastases may follow distinct mechanisms and growth kinetics. Animal studies comparing bone metastases of ER+ MCF7 cell and TN MDA-231 cell disclosed dichotomous patterns at early colonization. Whereas MDA-231 cells closely interact with osteoclasts throughout the colonization process, MCF7 lesions are initially interacting

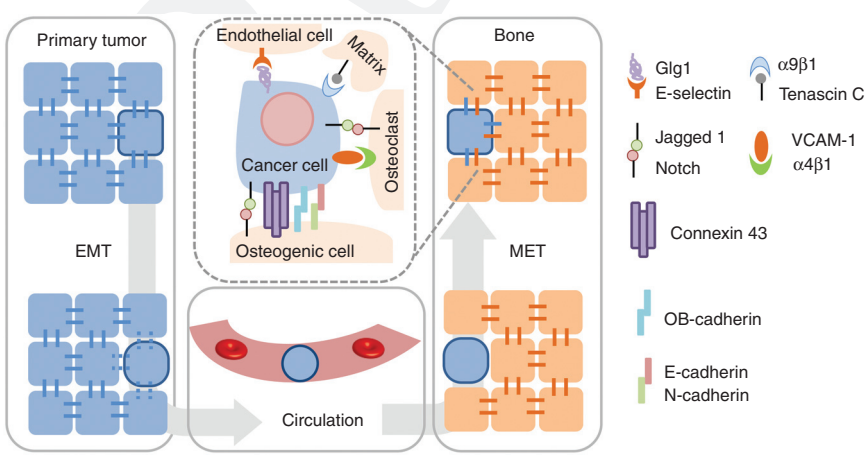

Figure 1. Epithelial-to-mesenchymal/mesenchymal-to-epithelial transition and functional molecules for attachment in early bone colonization. After cancer cells undergo EMT to facilitate their distal migration, they have to retrieve the epithelial trait for successful colonization in bone. During this process, cancer cells express different attachment proteins to form a physical connection with multitypes of resident cells.

with osteogenic cells (mainly MSCs and osteoblasts) before osteolytic growth starts (Lu et al. 2011; Wang et al. 2017). The distinct dependency on the osteogenic niche in different cancer cells might explain the inconsistent roles of osteogenic cells observed in different publications (Wang et al. 2015; Rossnag et al. 2018; Ren et al. 2019). Consequently, MDA-231 shows faster progression in bone than MCF7, which is in line with the much more rapid growth kinetics in the bone metastases of ER− breast cancer in the clinic (Zhang et al. 2009; Kennecke et al. 2010).

Overt Proliferation

Osteolytic Growth

The paradigm of late-stage, osteolytic bone colonization can be summarized as "osteolytic vicious cycle." It refers to the mutual activation between cancer cells and osteoclasts: while cancer cells can, directly and indirectly, promote osteoclast maturation, osteoclasts drive osteolysis, which releases growth factors embedded in the bone matrix and reciprocally fuel cancer cell proliferation (Guise et al. 2006; Weilbaecher et al. 2011). Multiple pathways have been implicated in this cycle. Specifically, cancer cells secrete factors such as parathyroid hormone (PTH) or PTH-related peptide (PTHrP), interleukin (IL-1, IL-6, and IL-11), TNF-α, which act on osteoblasts to modulate the expression of genes including RANKL (up-regulation) and OPG (down-regulation) via NFκB pathway (Cappellen et al. 2002). Macrophage-stimulating protein (MSP), CCL2 (Sørlie et al. 2002; Andrade et al. 2018), VEGF-A (Park et al. 2012) as well as extracellular matrixproteinases and transcriptional factors like GLI2 also regulate the osteolytic vicious cycle (Alexaki et al. 2010). The augments of these factors, in turn, boost osteoclast maturation and accelerate bone resorption, leading to widespread bone destruction. In this process, many growth factors deposited in the bone matrix—including TGF-β, IGF1, EGF, and Calcium ions—are released and reciprocally stimulate tumor growth (Fig. 2, left; Mundy 2002; Park et al. 2007).

Kang et al. (2003) conducted an unbiased study to identify genes driving bone metastasis

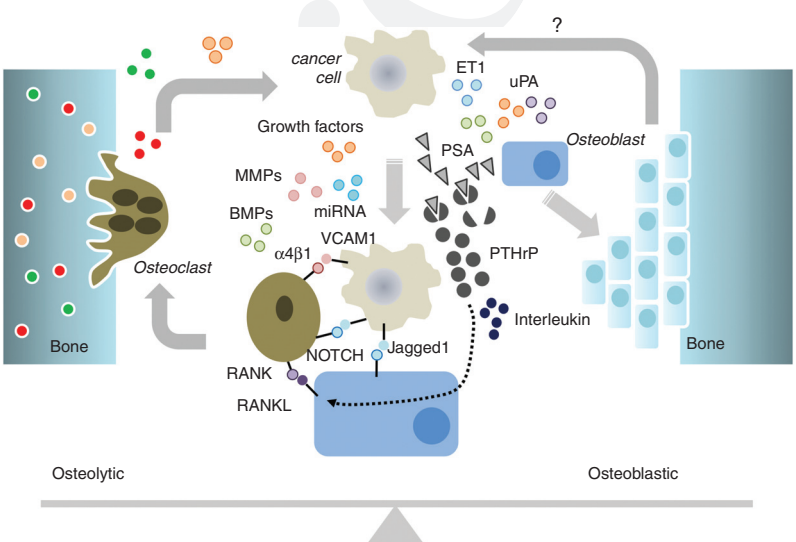

Figure 2. Overt osteolytic or osteoblastic progression in bone. Cancer cells give rise to osteolytic (*left*) or osteoblastic (*right*) outgrowth in bone. The osteolytic metastasis is fueled by reciprocal interactions between cancer cells, osteoblasts, and osteoclasts. This causes bone destruction and tumor progression, which is referred as a "vicious cycle." The mechanism of osteoblastic metastasis is yet to be further developed. The determinants that tilt the osteoblast–osteoclast balance are not fully understood, either.

through in vivo selection on triple-negative breast cancer cells. The major discoveries include chemokine receptor CXCR4 for bone-homing, angiogenic factor fibroblast growth factor-5 (FGF5), osteoclast stimulating factor interleukin-11 (IL-11), osteopontin (OPN), matrix metalloproteinase-1 (MMP1), integrins, and metalloproteinase with thrombospondin motifs 1 (ADAMTS1). The functions of these genes converge on activation of osteoclasts and the vicious cycle, supporting the central role of this process in late-stage bone colonization. One remaining question is whether the expression of these osteolysis-promoting genes is inherent in primary tumors or gradually acquired during the metastatic progression. The study of single-cell progenies of MDA-231 suggested that the osteolysis propensity was genetically determined before cancer cells arrive in bone (Minn et al. 2005). Nevertheless, we cannot rule out the possibility that alternative mechanisms such as epigenetic reprogramming may also contribute to the acquisition of osteolytic phenotype, especially for those indolent bone metastases (Valastyan and Weinberg 2011).

Beyond the protein-coding genes in bone metastases, Ell et al. (2013) unveiled a group of tumor-induced miRNAs as regulators and biomarkers of osteolytic bone metastasis. Specifically, the expression of miR-33a-5p, miR-133a, miR-141-3p, miR-190, and miR-219-5p were found to be suppressive for bone metastases by targeting osteoclast differentiation genes. In contrast, miR-16 and miR-378, which are up-regulated during osteoclastogenesis, were proposed to serve as serum biomarkers for diagnosis of bone metastasis in breast cancer.

Osteoblastic Growth

Osteoblastic bone metastases, characterized by abnormal new bone growth, occur mostly in prostate cancer. Many molecules and pathways mediate both osteoblastic prostate cancer and osteolytic breast cancer, such as TGF-β, fibroblast (FGF), insulin-like (IGF), vascular endothelial (VEGF), and platelet-derived (PDGF) growth factors, Wnt signaling, and bone morphogenetic proteins (BMPs) (Obenauf and Massagué 2015). In fact, this is expected because osteoblast activities are also required in osteolytic metastases, and most of these pathways regulate the growth and differentiation of osteoblasts. Only a few factors are known to be uniquely needed by osteoblastic bone metastases, which will be discussed below. However, the determinants that tilt the osteoblast–osteoclast balance and cause the discrepancy between overt osteoblastic and osteolytic metastases are yet to be identified (Fig. 2, right).

PSA: The prostate surface antigen (PSA) is an important serum biomarker used to monitor prostate cancer tumorigenesis as well as metastatic recurrence (Manca et al. 2017). Active PSA in the bone microenvironment enhances tumor growth by altering signaling specifically in osteoblasts including up-regulation of TGF-β and RANKL, down-regulation of osteoprotegerin, enhancement of Runx2 expression (a transcription factor essential for osteoblast differentiation) and elevation of the Wnt signaling (Chirgwin and Guise 2006). Particularly, PSA cleaves PTHrP, disrupts the indigenous bone resorption, and in turn, allows the enrichment of osteoblasts, which may drive the osteoblastic metastases instead of the osteolytic outgrowth (Iwamura et al. 1996).

Endothelin 1 (ET1): Endothelin-1 is reported to stimulate bone formation and osteoblast proliferation. A strong correlation between the ET1 expression and osteoblastic bone metastases is observed in prostate cancer patients (Nelson et al. 1995). Interestingly, even in breast cancer bone metastases, which are usually osteolytic, the expression of ET1 predisposes an osteoblastic growth (Yin et al. 2003).

Osteomimicry: The Metabolic Adaptation

Recently a growing body of evidence shows that cancer cells rewire their metabolic program to adopt the metastatic organs. Upon colonization, cancer cells tend to resemble the metabolism of local tissues for survival, which is often associated with the adaption to the energy resource, nutrition availability and oxygen level at the metastatic organs (Schild et al. 2018). In bone metastasis, this specific adaption process is

termed as osteomimicry, which is proposed to mimic the metabolism of resident cells in bone, especially osteoblast. Osteoblasts appear to metabolize glucose ~~mostly~~ into lactate even in the presence of sufficient oxygen, a process known as aerobic glycolysis or Warburg effect (Karner and Long 2018). Similarly, bone metastases of breast cancer cells are also observed to increase the glycose uptake by up-regulation of glucose transporters (e.g., GLUT1) and release a larger amount of lactate—the products of aerobic glycolysis, compared with lesions in other organs (Hanahan and Weinberg 2011; Lemma et al. 2017). Consistently, pharmacological inhibition of the lactate transporter MCT-1 impairs lactate-induced bone resorption and hence blunts the bone metastases progression.

The reprogrammed metabolism in bone metastases is further exemplified by the high expression of OPN. OPN helps anchor osteoclasts to the mineral matrix of bone and activate glucose and lipid signaling (Shi et al. 2015), indicating an important role in sugar homeostasis and glucose metabolism in the skeleton. The up-regulation of OPN was observed in bone metastases of advanced nasopharyngeal carcinoma as well as breast cancer, and may predict bone metastases in other cancer types (Carlinfante et al. 2003; Kruger et al. 2014; Hou et al. 2015).

Escaping the Immune Surveillance

The role of bone marrow-derived cells, especially the immune cells in metastasis regulation, was long overlooked due to limited clinical samples and lack of suitable experimental models. Bone represents a specific microenvironment in which microorganism infection is less common but bone repair and regeneration are constantly ongoing. Therefore, it has been traditionally speculated that the innate immune response other than the adaptive response may dominate in the bone microenvironment (Charles and Nakamura 2014). Additionally, the bone microenvironment contains very high numbers of MDSCs and immunosuppressive regulatory T cells (Treg), thereby making bone a permissive environment for DTCs to hide from immunosurveillance (Fujisaki et al. 2011; Zhao et al. 2012). The local immune response might be further blunted as DTCs hijack HSC niches for survival, because the HSC niche is immune-privileged, offering protection from immunological insults (Fujisaki et al. 2011).

Despite the potential immune privilege of the bone environment, evidence also emerges to suggest possible roles of adaptive immune cells in bone metastasis. This is exemplified by a clinical breast cancer analysis highlighting the enrichment of CD56+ CD8+ T cells and memory CD4+ T cells in bone marrow aspirates when DTCs are present in the patients (Feuerer et al. 2001). In another study, depletion of the infiltrating plasmacytoid dendritic cells (pDCs) dampens the development of bone metastasis by expanding CD8+ T cells (Bidwell et al. 2012; Sawant et al. 2012). These results urge for a more thorough characterization of the immune

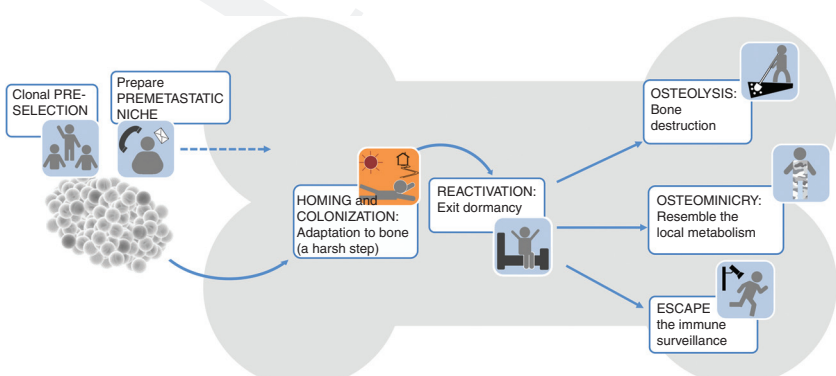

Figure 3. Metastatic traits for bone colonization.

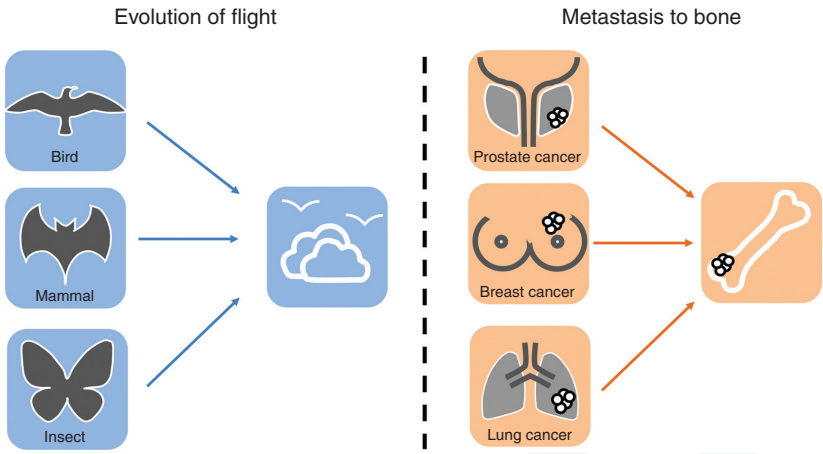

Figure 4. Bone metastases from different origins are analogous of convergent evolution. (*Left*) Seagull, bat, and butterfly independently evolved the capacity of flight based on distinct structures of wings. (*Right*) Likewise, cancer cells originated from different organs may acquire comparable capacity for bone colonization underlying different molecular mechanisms.

landscape and a deeper understanding of the functional roles of various immune cells in different stages of bone colonization.

CONCLUDING REMARKS

Bone tropism is about how cancer cells adapt to bone microenvironment in every key step of bone colonization. The specificity of bone environment exerts unique selection pressure on metastatic cells and hence endows them with special traits and phenotypes for outgrowth in bone (Fig. 3). While the conceptual framework of bone metastasis is well established, the complexities at the molecular level are frequently observed in different biological contexts. This prompts us to develop new insight to synthesize and integrate the existing knowledge.

Perhaps one useful way to reflect on bone tropism is to use convergent evolution as an analogy: the functionally similar physiological traits may be selected based on structures of different biological ontogeny. For instance, in the evolution of flight, animals from different ancestors independently evolve the "wing structure" to fly (Stayton 2015). Likewise, cancer cells originated from distinct cancer types may be selected to acquire similar traits to colonize bone. Similar to different structural solutions of the wing in different species, cancer cells evolve to use the different toolkit to build up their bone-tropic traits at the molecular level (Fig. 4). This analogy might help us better understand the commonality and diversity of metastatic mechanisms across different cell models and cancer types. Indeed, recent studies have started to characterize metastases from perspectives of evolutionary biology (Turajlic and Swanton 2016; Ullah et al. 2018). These new perspectives may help us nominate the major and common players in bone metastases, accelerate the translation to clinical practice, and revolutionize our understanding of these devastating diseases.

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Queries

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