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Epigenetic control of the vicious cycle

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HIGHLIGHTS

- DNA hypo- and hyper-methylation regulate tumor suppressors and oncogenes that may influence bone metastatic progression.
- Hypermethylation of H3K27me3 promotes stemness and metastatic spread, and blocking this histone modification reduces bone metastasis and subsequent seeding of other metastatic sites.
- Histone deacetylase (HDAC) inhibitors reduce the growth of solid tumors in the primary site and promote dormancy, but increase bone metastasis through osteoclast-mediated bone resorption.
- Drugs that target epigenetic modifications are not specific, so careful monitoring of tumor progression at all metastatic sites is critical in the clinical setting.

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ABSTRACT

Epigenetic alterations, including DNA methylation and post translational modifications to histones, drive tumorigenesis and metastatic progression. In the context of bone metastasis, epigenetic modifications in tumor cells can modulate dissemination of cancer cells to the bone, tumor progression in the bone marrow, and may be associated with patient survival rates. Bone disseminated tumor cells may enter a dormant state or stimulate osteolysis through the "vicious cycle" of bone metastasis where bone disseminated tumor cells disrupt the bone microenvironment, which fuels tumor progression. Epigenetic alterations may either exacerbate or abrogate the vicious cycle by regulating tumor suppressors and oncogenes, which alter proliferation of bone-metastatic cancer cells. This review focuses on the specific epigenetic alterations that regulate bone metastasis, including DNA methylation, histone methylation, and histone acetylation. Here, we summarize key findings from researchers identifying epigenetic changes that drive tumor progression in the bone, along with pre-clinical and clinical studies investigating the utility of targeting aberrant epigenetic alterations to treat bone metastatic cancer.

1. Introduction

Epigenetics is the study of dynamic and heritable genomic modifications that do not alter the DNA sequence. Examples of epigenetic modifications include DNA methylation and post translational modifications to histones, which drive tumor initiation, progression, and metastasis [1]. Many cancers including breast, prostate, lung, melanoma, and multiple myeloma metastasize to the bone [2], and progression of these cancers has been linked to epigenetic modifications.

Metastatic cancer cells often disseminate to the bone where they can reside and eventually contribute to recurrence [2,3]. Disseminated tumor cells remain dormant in the bone, often for months or years, until reactivating and developing into overt, clinically detectable metastases. The exact mechanisms regulating the reactivation of dormant tumor cells in the bone is not completely understood. Once tumor cells colonize the bone, they disrupt bone homeostasis by secreting a variety of molecules including parathyroid hormone-related protein (PTHrP), vascular endothelial growth factor (VEGF), and interleukin-11 (IL-11), which stimulate the receptor activator of NFκB ligand (RANKL)-mediated activation of osteoclasts and bone resorption. When osteoclasts resorb the bone matrix, mitogenic factors including insulin-like growth factor-1 (IGF-1) and transforming growth factor-β (TFG-β) are released, which promotes tumor cell proliferation and further secretion of osteolytic factors, thus establishing a "vicious cycle" of tumor progression and tumor-induced bone loss [2]. Understanding the regulation of the vicious cycle is paramount to successfully targeting bone metastatic

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cancers.

This review is focused on the epigenetic alterations that drive the vicious cycle of bone metastasis and potential vulnerabilities to treat patients with bone metastatic cancer. Here, we review the epigenetic regulation of bone metastasis in multiple tumor types with an emphasis on breast cancer, given that most of the research in epigenetics of bone metastasis has been in the context of breast cancer.

2. DNA methylation

DNA methylation is one of the most studied epigenetic modifications and occurs predominantly in CpG islands preferentially located at the promoter of more than 50% of human genes. DNA methylation is typically responsible for gene silencing by covalently adding methyl groups from S-adenosylmethionine (SAM) to the 5 position of the cytosine pyrimidine ring. This prevents transcription factors from binding to DNA and leads to the recruitment of methyl-binding domain proteins, which associate with histone modifications to reconfigure the chromatin to repress gene expression [1]. Promoter methylation and reduced expression of the tumor suppressor genes high in normal 1 (*HIN-1)* and retinoic acid receptor beta (*RAR-β),* which regulate cell growth and invasion, is observed in bone metastatic breast cancer patient biopsies compared to normal breast tissue [4]. Additionally, *HIN-1* methylation is associated with an increased risk of bone metastasis. These results suggest that promoter methylation may silence expression of tumor suppressors, increasing proliferation of breast cancer metastases in the bone, but the microenvironmental mechanisms that cause these tumor suppressors to become methylated in bone metastatic cancer remains unclear. Methylation of the androgen receptor (AR) promoter has also been identified as a marker of therapy resistance and is associated with poor outcomes in prostate cancer [5]. Researchers analyzed genome-wide promoter associated CpG methylation signatures from 94 total breast tumor samples, including bone metastases, and identified a Methylation Classifier for Androgen receptor activity (MCA) signature. MCA negative patients exhibited hypermethylation of AR-associated genes and a worse prognosis after androgen deprivation therapy (ADT) [5]. Thus, determining promoter methylation and MCA signatures in cancer patients with bone metastases may be useful in predicting prognosis and selecting clinical therapies. Uncovering mechanisms to reduce promoter methylation to regain expression of tumor suppressor genes remains an active area of pre-clinical investigation as a therapeutic strategy to treat bone metastatic tumors.

DNA methylation can also occur at transcriptional enhancer regions. By analyzing the DNA methylation profiles from thousands of melanoma tumors, researchers have identified enhancers as the most differentially methylated region of the genome when comparing normal tissue, primary tumors, and metastases [6]. Specifically, hypomethylation is enriched in the enhancer regions of the oncogenes *CTYL1* and *KIF14*, which are upregulated in bone metastatic melanoma patient samples but not in primary tumors. When DNA methylation is inhibited using 5-azacitidine *in vitro*, *CTYL1* and *KIF14* are induced in bone metastatic melanoma cells. Additionally, when melanoma cells from the primary tumor are cultured with primary human osteoblasts, the expression of *CTYL1* and *KIF14* increases compared to melanoma cells cultured alone [6]. These results suggest that the bone microenvironment induces epigenetic changes in metastatic cells that may increase the expression of oncogenes and stimulate proliferation of bone disseminated tumor cells.

DNA methylation is maintained by DNA methyltransferases (DNMTs), which are encoded by genes including *DNMT1, DNMT2, DNMT3A,* and *DNMT3B.* To determine if changes in the levels of DNMTs contribute to increased promoter methylation of bone metastatic breast cancer cells, a study investigated DNMT1 expression in 31 cases of bone metastatic invasive ductal carcinoma and found that DNMT1 is expressed in tumor cells but not stromal cells, and DNMT1 is lowest in bone metastatic tumor cells compared to brain metastases [7]. However, DNMT1 positivity is associated with shorter overall survival for patients with bone metastases. These findings provide a potential enzymatic mechanism for the increased promoter methylation observed in bone metastatic breast cancer cells and implicate DNMT1 as a driver of high-risk bone metastatic breast cancer. The expression level of other DNMTs was not evaluated in this study; however, defining the expression of DNMT proteins in bone metastatic tumor types other than breast cancer may offer additional therapeutic strategies to target bone metastases. In addition to DNMTs, DNA methylation is regulated by SAM, a ubiquitous methyl donor that prevents DNA hypomethylation. In MDA-MB-231 human breast cancer cells, SAM induces the downregulation of oncogenes and pro-metastatic genes including *MUC1, uPa, FABP7*, and epithelial-to-mesenchymal transition (EMT) associated genes *SPARC*, *HAS2*, *HAS3* and *SOX4* [8]. Although this study did not explore potential effects on bone metastasis, preclinical studies in bone metastatic prostate cancer show that SAM-treated PC-3 human prostate cancer cells form significantly smaller skeletal lesions when injected into the tibia of immunocompromised mice compared to untreated cells [9]. These findings suggest the utility of DNA hypermethylation pharmacological agents to reduce the development and progression of skeletal metastasis by reducing DNA hypomethylation, but the specific targets of these therapies will need to be identified for each tumor type, since hypermethylation of tumor suppressors would be counter-productive.

While multiple studies implicate gene repression by DNA hypermethylation in bone metastatic cancer progression, DNA hypomethylation is also reported to promote progression of bone metastasis by increasing the expression of oncogenes and pro-metastatic genes. IL-1β is a pro-inflammatory cytokine that is associated with an increased risk for developing bone metastasis [9]. Expression of IL-1β in MCF7 human breast cancer cells increases tumor progression and depends on the demethylating actions of ten-eleven translocation proteins (TETs). Inhibition of TET enzymes interfere with EMT and reduce markers of bone metastasis including versican, osteopontin, and prolactin receptor [10]. These studies highlight the role of DNA hypomethylation as an essential regulator of oncogene expression in bone metastatic breast cancer and suggest that inhibiting DNA hypomethylation may be a therapeutic strategy to reduce bone metastasis. This idea is contradictory to previous studies which showed that promoter hypermethylation promotes bone metastatic breast cancer growth by decreasing the expression of tumor suppressor genes including *HIN-1* and *RAR-β* [4]. Together, these studies highlight the need for further preclinical testing of DNA demethylating and hypermethylating agents, including large-scale analyses of transcriptional targets and functional assays, to determine how best these drugs may be used to prevent or inhibit bone metastatic tumor growth.

2.1. Clinical targeting of DNA methylation

DNA hypermethylation inhibitors are currently under investigation as a therapeutic target for advanced solid tumors, including metastatic breast cancer. NCT00359606 is a phase I trial using intravenous 5-fluoro-2′-deoxycytidine (5-fluoro-2-deoxycytidine) (FdCyd), a DNA methyltransferase inhibitor, paired with the cytidine deaminase inhibitor tetrahydrouridine (THU). Given previous findings highlighting the important role of DNA hypermethylation in metastasis and tumor progression [4–10], investigators hypothesized that FdCyd would be more successful at treating advanced solid tumors, including metastatic breast cancer, than THU alone. However, recent studies highlight the role of DNA hypomethylation in elevated expression of oncogenes associated with bone metastasis [9,10]. Thus, it will be essential to closely monitor patients receiving DNMT inhibitors such as FdCyd to ensure that inhibition of DNMTs does not induce hypomethylation of pro-metastatic oncogenes that result in progression of bone metastatic breast cancer.

3. Histone modifications

Histone modifications, including acetylation and methylation,

regulate gene expression by controlling the accessibility of chromatin to transcription factors and RNA transcription machinery. While several groups have studied DNA methylation in bone metastatic tumors, most of the work on understanding the epigenetic regulation of the vicious cycle of cancer metastasis in the bone has been focused on histone modifications.

3.1. Histone methylation

Histone methylation occurs on lysine and arginine residues and is maintained by histone methyltransferase (HMT) enzymes. Lysine residues are mono-, di-, and trimethylated, while arginine residues undergo monomethylation or symmetric or asymmetric dimethylation [11]. The effect of histone methylation depends on the targeted residues. For example, methylation of lysine 4 of histone H4 (H3K4) typically activates transcription, while methylation of H3K9 or H3K27 is typically repressive [1]. Thus, it is essential to identify the target histones and residues of different HMTs when considering potential therapeutic vulnerabilities.

Recently, investigators determined EZH2, the catalytic subunit of polycomb repressive complex 2 (PRC2), is essential for bone metastatic breast cancer progression [12]. EZH2 catalyzes the trimethylation of histone H3K27 (H3K27me3) which compacts chromatin, causing transcriptional repression of downstream genes. EZH2 activity mediates epigenetic reprogramming of bone-disseminated breast and prostate cancer cells, increasing stemness and enhancing metastatic spread to other tissues. These reprogrammed bone metastatic cells can give rise to multi-organ metastases, highlighting the metastasis-promoting effect of enhanced EZH2 activity. Additionally, EZH2 inhibition decreases stemness and plasticity of bone-disseminated tumor cells, which dramatically reduces secondary metastases [12]. The EZH2 inhibitor tazemetostat is currently under investigation for the treatment of multiple tumor types including metastatic melanoma (NCT04917042, NCT04557956, NCT03028103).

3.2. Histone acetylation

Histone acetylation plays an essential role in differentiation and cell cycle progression by opening chromatin and activating transcription. The bromodomain and extra terminal domain (BET) family of proteins induces the expression of oncogenes by recruiting transcription factors and coactivators to acetylated lysines of histones. JQ1 is a small molecule inhibitor of BRD4, which is a member of the BET protein family. Treatment of osteosarcoma patient cells *in vitro* with JQ1 decreases expression of the oncogenes *MYC, CDK4, CDK6,* and *RUNX2* by depleting BRD4 at the promoter site, which reduces proliferation of osteosarcoma tumor cells [13,14]. JQ1 also inhibits osteoclasts by preventing BRD4-dependent RANKL induction of NFATC1 [13], making JQ1 an ideal therapeutic to target bone metastases and tumor-induced bone destruction. Recent studies show that JQ1 works synergistically with icaritin, which inhibits osteoclast differentiation, to treat bone metastatic breast cancer [14]. Collectively these data are promising, and further studies are warranted to determine the effect of JQ1 on other tumor types that disseminate to the bone.

Acetylation of histones at the ε-amino group of lysine residues is regulated by the balance of histone acetyltransferase (HAT) and histone deacetylase (HDAC) activity. Lysine acetylation by HATs weakens the interactions between neighboring nucleosomes, which relaxes the chromatin structure and increases the accessibility of DNA for transcription. In contrast, HDACs deacetylate histones, which strengthens the interactions between nucleosomes and represses transcription. Disruption of normal levels of acetylation by HATs and HDACs is known to drive tumorigenesis and metastasis by decreasing the expression of tumor suppressors and increasing the expression of oncogenes and pro-metastatic genes [15]. The HDAC inhibitor trichostatin A (TSA) prevents c-Src activation of Ets1 and the binding of Ets1 to the CXCR4 promoter, which interferes with homing, angiogenesis and survival of bone metastatic breast cancer cells in a c-Src-dependent manner [16]. These findings suggest that c-Src might be a biomarker to predict the sensitivity patterns of bone metastatic breast cancer to HDAC inhibitors.

HDAC inhibitors are FDA-approved for clinical use to treat multiple blood cancers. There has been considerable interest in using these inhibitors for the treatment of solid tumors. In breast cancer, HDAC inhibition successfully reduces proliferation in the primary tumor site, in part by stimulating the breast tumor suppressor and metastasis suppressor leukemia inhibitory factor receptor (LIFR), which promotes dormancy [17,18]. These data suggest that HDAC inhibitors may be an effective way to target bone metastatic breast cancer. As such, clinical trials have aimed to improve the outcome for patients with advanced breast cancer by incorporating the HDAC inhibitor entinostat with standard of care chemotherapy. A comprehensive review of different classes of HDAC inhibitors currently in clinical trials for the treatment of bone and lung metastatic tumors was previously published [15]. Notably, a phase III study demonstrated that patients with breast cancer receiving HDAC inhibitors did not gain an increase in survival, and they experienced adverse events as a result of HDAC inhibitor therapy [19]. After the results of this study were published, other clinical trials involving entinostat for advanced breast cancer patients were suspended [20,21]. The finding that HDAC inhibitors do not improve outcomes in patients with metastatic disease is initially surprising, especially given that HDAC inhibitors promote dormancy and increase LIFR expression even when it is down-regulated by pro-tumorigenic bone microenvironment-induced factors, like elevated expression of PTHrP or hypoxia [22]; however, subsequent pre-clinical studies investigating the impact of HDAC inhibitors on bone metastatic progression may shed some light on the clinical findings. In pre-clinical breast cancer bone metastasis models, HDAC inhibitors increase osteolysis and the incidence and progression of bone metastases [23], in stark contrast to the anti-tumor effects of HDAC inhibitors at the primary tumor site [17]. However, the negative effect of HDAC inhibitors on bone metastasis is reversed when combined with an anti-resorptive bisphosphonate [23], suggesting that HDAC inhibitors stimulate bone remodeling and osteoclast-mediated bone resorption, which is consistent with previous reports of HDAC inhibitor effects on bone remodeling [24]. Based on these findings, patients co-administered entinostat and a bisphosphonate may experience a reduction in tumor burden without an increase in osteolysis and adverse events related to increased bone remodeling, but in the absence of a bisphosphonate, HDAC inhibitors may fuel bone metastatic progression. These data suggest that coadministration of a bisphosphonate with HDAC inhibitors may be essential to prevent bone relapse in patients with solid tumors at risk of developing bone metastases. While further clinical trials incorporating bisphosphonates would be interesting and may benefit patients with advanced bone metastatic cancers, the field is hesitant to initiate further clinical trials using this epigenetic therapy based on the results from the phase III study that failed to meet its primary endpoint of progression free survival in patients with metastatic breast cancer [19].

4. Final thoughts

Many patients with cancer experience bone metastases, which are a significant cause of morbidity and mortality. The exact epigenetic alterations and chromatin conformation changes that regulate each step of the vicious cycle are not well defined, but multiple studies demonstrate epigenetics impact bone metastasis and tumor progression in bone (Fig. 1). More research into the epigenetic regulators of bone metastasis is required to discover additional therapeutic vulnerabilities to improve outcomes for patients with bone metastatic cancer. When designing clinical trials involving epigenetic regulators, it is important to consider findings from pre-clinical studies. For example, the use of DNMT inhibitors like FyCyd is currently in clinical trials for the treatment of advanced breast cancer (NCT00359606), but this may cause

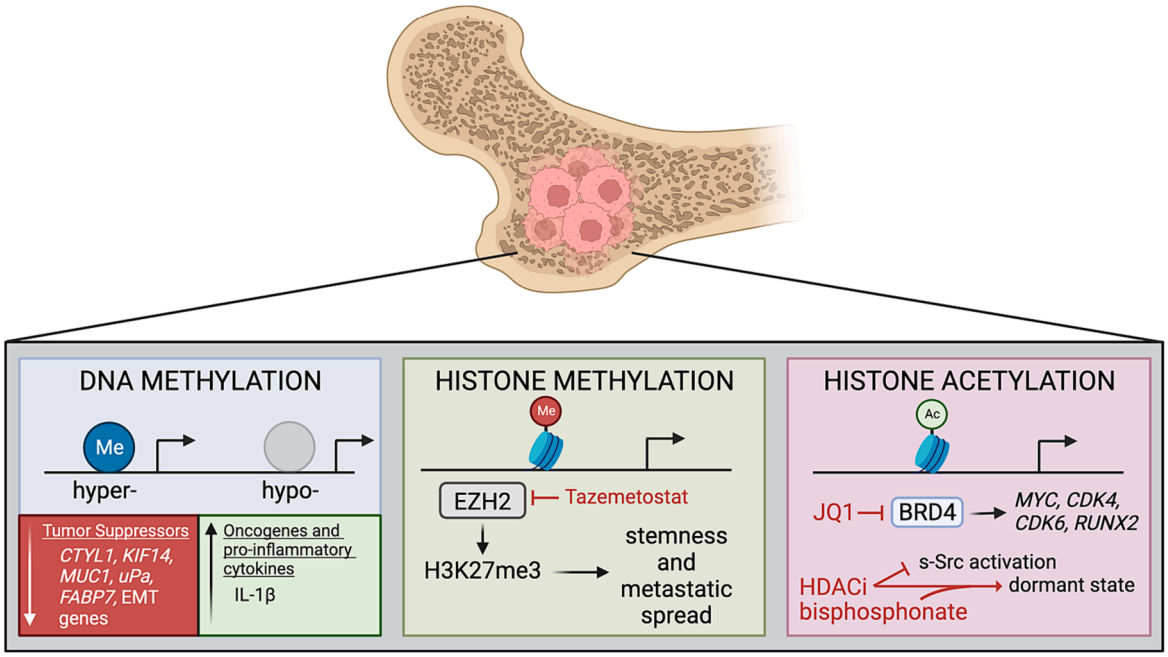

Fig. 1. Epigenetic modifictions that drive bone metastasis. (Top) Metastatic tumor shown in pink within the bone and (bottom) types of epigenetic modifications that drive bone metastasis. (Left) DNA hypermethylation, shown with a blue circle in front of the transcription start site, decreases the expression of tumor suppressors including *CTYL1, KIF14, MUC1, uPa, FABP7*, and EMT genes that drive bone metastases. DNA hypomethylation, shown with an empty grey circle before the transcription start site, leads to increased expression of oncogenes and pro-inflammatory cytokines like IL-1β which increase the proliferation of bone metastatic tumor cells. (Middle) EZH2, the catalytic subunit of polycomb repressive complex 2, catalyzes the trimethylation of histone H3K27 causing transcriptional repression of downstream genes which increases stemness and enhances metastatic spread to other tissues. This metastatic spread can be prevented by inhibiting EZH2 with Tazemetostat. (Right) BRD4 activates transcription of oncogenes including *MYC, CDK4, CDK6,* and *RUNX2* by recruiting transcription factors to acetylated lysines, thus promoting proliferation of bone metastases. Additionally, inhibiting histone acetylation with HDAC inhibitors paired with bisphosphonate may be a therapeutic option for patients with bone metastatic tumors by inhibiting s-Src activation and forcing bone metastatic tumor cells to a dormant state. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hypomethylation resulting in the expression of oncogenes, similar to TET enzymes that induce tumor proliferation [10]. Additionally, preclinical studies suggest that HDAC inhibition may have a clinical benefit in bone metastatic breast cancer if patients receive a bisphosphonate to reduce bone resorption. Since HDAC inhibitors have a well-established effect on bone mass, it will be crucial to determine whether other epigenetic modifiers similarly disrupt bone microarchitecture, and how this might impact their ability to target bone metastatic cancers.

CRediT authorship contribution statement

Madeline B. Searcy: Conceptualization, Visualization, Writing – original draft, Writing – review & editing. **Rachelle W. Johnson:** Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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