Mechanisms of breast cancer dormancy in bone metastasis

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REVIEW

Mechanisms of breast cancer dormancy in bone metastasis

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Abstract
Bone metastasis remains a serious threat to breast cancer patients. This condition arises from the outgrowth of previously dormant tumour cells in this site. Dormant tumour cells are almost impossible to detect in human patents, and these cells acquire "stem like" characteristics rendering them resistant to current cancer therapies. Furthermore, the development of therapies to target this population has proved challenging. The bone marrow is a particularly permissive environment for tumour cell dissemination and dormancy, but the mechanisms regulating this process remain to be completely elucidated. Expansion of our understanding of the mechanisms underlying tumour dormancy is critical to the development of targeted therapies and thus, the prevention, or treatment, of metastatic disease. This review aims to explore mechanisms of tumour dormancy in bone, in detail, focusing specifically on breast cancer dormancy. In addition to subsequent discussion of traditional and new, state of the art, methods of studying dormancy to aid further research efforts.

Keywords Breast cancer · Bone microenvironment · Cancer dormancy · Dormant tumour cells · Metastatic disease · Tumour dormancy models

Introduction

Metastatic disease is a considerable clinical challenge and is a major cause of fatality in breast cancer patients. The process of metastasis is poorly understood, and patients can often relapse months, years, or decades after removal of the primary tumour. Cancer progression to metastases and dormancy requires a series of key stepwise changes, regulated by the microenvironment. Cells must undergo epithelial-to-mesenchymal transition (EMT) to allow for entry into the vasculature. Circulating tumour cells (CTCs) home to bone via specific signalling axes, adhere to stromal cells and undergo mesenchymal-to-epithelial transition (MET) and extravasation. From here, disseminated tumour cells (DTCs) can have different fates: cell death, dormancy, or further proliferation into metastatic colonies [1–4].

Tumour cell dormancy within bone allows DTCs to reside in a quiescent state for prolonged periods, resistant to therapeutics, with latency periods exhibiting varying lengths, spanning from months to decades [5–7]. Bone metastasis secondary to breast cancer occurs in 10–30% of cases [8, 9], yet the presence of DTCs in the bone marrow is detected in > 60% of patients when autopsied, with tumour dissemination in distal organs thought to occur before clinical detection of the primary tumour [10–16]. Interestingly, non-proliferative DTCs can survive in the bone marrow simultaneous to other organs containing proliferative metastases without becoming proliferative themselves, demonstrating the importance of the tumour microenvironment in regulating dormancy [17]. There are many factors and pathways that have been implicated in the induction of breast cancer tumour dormancy in bone, or reactivation of dormant cells, but key mechanisms remain to be identified, with studies struggling to effectively replicate the dormant microenvironment and often relying on pre-clinical models [2, 5, 18]. This review will outline the current understanding of mechanisms of breast cancer dissemination to the bone, the impact of the bone microenvironment on tumour cells dormancy and the current tools being utilised to study the process, bringing together knowledge in a rapidly advancing field of cancer research.

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Tumour cell dissemination

Within bone, the different microenvironmental niches act distinctly to impact tumour cell dissemination, dormancy and tumour growth. Many tumours utilise the perivascular niche and haematopoietic stem cell (HSC) homing pathway as footholds to infiltrate the bone marrow. Breast cancer cells have been shown to home to highly osteoblast-populated areas. Anti-resorptive therapy, zoledronic acid (ZOL), alters the endosteal niche and results in breast cancer migration into alternative, osteoblast-rich areas of bone [19], driven by the CXCL12:CXCR4 signalling axis (Fig. 1). Osteoblastic expression of CXCL12 is coupled with CXCR4 expression commonly observed in metastatic breast cancer cells [20–22]. The primary role of CXCL12 in these environments is thought to be establishing the cancer stem cell (CSC) niche, promoting cell survival, proliferation, angiogenesis and metastasis [21, 23, 24]. CXCL12 knock-down in breast cancer cells (BCCs) prevents contact between cells and the bone marrow stroma [25]. E-selectin interactions appear to be key for allowing breast cancer cell entry into the bone marrow, with CXCL12/CXCR4 interactions attaching BCCs to the perivascular niche, using the Wnt pathway, facilitating bone homing and metastasis [23, 26, 27]. In the same chemokine family, CXCL5 is associated with metastatic colonisation, particularly observed in BCCs in mouse models. CXCL5 signalling via its receptor, CXCR2, is sufficient to promote breast cancer cell proliferation and bone colonisation, with inhibition of CXCR2 blocking metastatic cell proliferation [28].

E-cadherin is a fundamental component in regulating metastatic spread, maintaining cell–cell junctions and preventing abnormal cell proliferation. The loss of E-cadherin via genetic or epigenetic silencing is indicative of invasive tumours [29, 30]. Snail family transcriptional repressor (SNAI) 1 and 2, zinc finger E-box binding homeobox (ZEB) 1 and 2, and E47 transcription factors are involved in the repression of E-cadherin, increasing metastatic tumour cell dissemination. In ER + breast cancer, ZEB1/2, vimentin (VIM) and fibronectin 1 (FN1) are upregulated by transcription factors that regulate EMT and maintain a dormant state [31, 32]. When comparing xenografts, triple negative breast cancer (TNBC) cells were shown to proliferate at similar rates in primary and secondary sites, whereas ER + DTCs proliferated slower, exhibited decreased CDH1 (E-cadherin) expression and increased ZEB1/2 expression, taking on a dormant state [31]. In bone specifically, E-cadherin negative breast cancer cells (MDA-MB-231) xenografts resulted in multiple bone metastatic lesions in mice, yet overexpression of E-cadherin resulted in an impaired capacity to form osteolytic metastases [33].

Integrins are cell-surface receptors that bind to components of the extracellular matrix (ECM) and aid in cell proliferation, differentiation, adhesion and migration [34, 35].

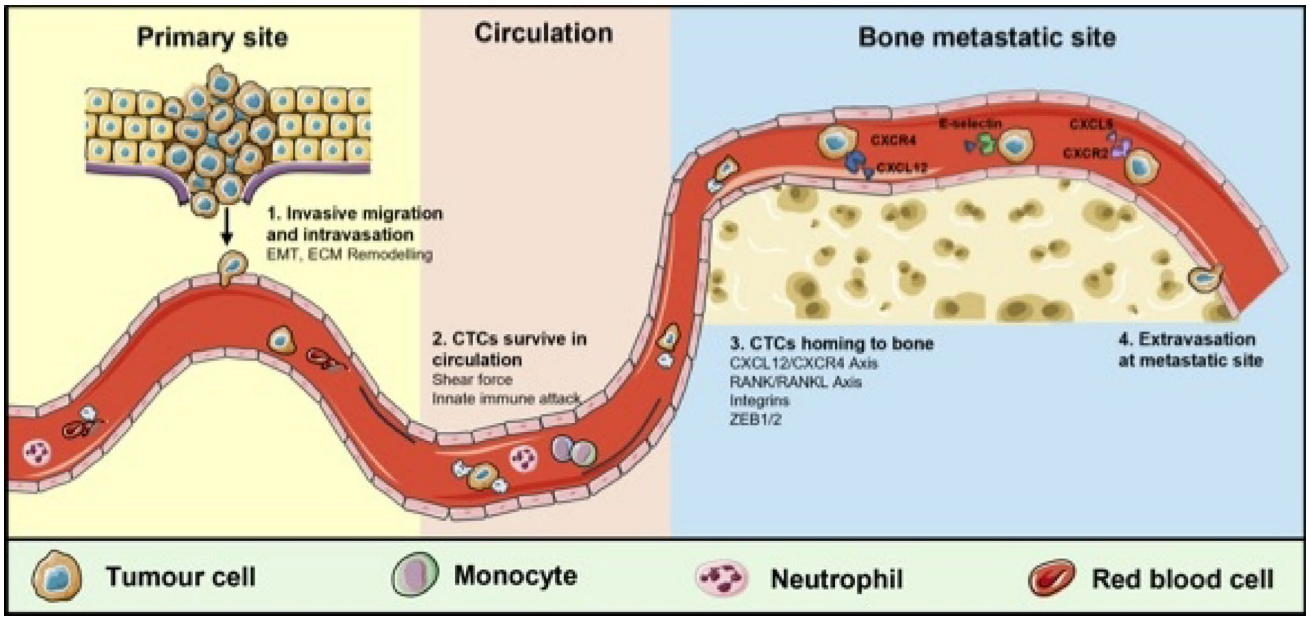

Fig. 1 Metastasising cells undergo dynamic changes to adapt to changing environments in what is known as the metastatic cascade [1–4]1. Tumour cells begin the process of metastasis, undergoing epithelial-to-mesenchymal transition (EMT) and remodelling of the extracellular matrix (ECM), before entering blood vessels via intravasation. 2. Circulating tumour cells (CTCs) survive a series of stress factors including matrix detachment, shear forces and immune system attack. 3. CTCs begin homing to the bone, using CXCL12/CXCR4, CXCL5/CXCR2 and E-selectin pathways. 4. CTCs undergo mesenchymal-to-epithelial transition (MET) and extravasation from the blood supply upon reaching the bone, now known as disseminated tumour cells (DTCs). Figure created with adapted images from Servier Medical Art (http://smart.servier.com), licensed under CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/).

Inhibition of integrin β1 results in decreased breast cancer cell migration and adhesion to human bone marrow stromal cells (hBMSCs) *in vitro* [36]. Bone metastasis was similarly reduced in mouse xenograft models exposed to integrin α5 inhibitor, GLPG0187 [37]. Integrin β3 is established to be a key factor in early bone metastasis with involvement in migration capacities in vitro and increased vascular dissemination in vivo. Down-regulation of tumour integrin β3 resulted in impaired bone metastasis of a mammary tumour [38], yet had no effect on lung metastases, highlighting the impact of the metastatic site [39]. Similarly, overexpression of this integrin resulted in increased bone metastasis in vivo following IV injection of the breast cancer cell line, MDA-MB-231, into mice [40]. Of note however, is that tumour integrin β3, rather than stromal integrin β3, proves to be the main driver of this increased migration and metastasis, suggesting the specific microenvironment may not be relevant to its actions [38].

Clinical evidence has shown a positive correlation between *RUNX2* expression and the development of breast cancer bone metastasis [41–43]. Further investigations revealed Runx2 acts to promote bone attraction and adhesion of breast cancer cells in an integrin α5-dependent manner, aiding in tumour cell dissemination [43]. Silencing of integrin α5 results in impaired tumour cell adhesion and migration to fibronectin matrices in vitro, reducing osteolytic bone metastases in vivo, whereas overexpression promoted bone metastasis in vivo [44]. Acting as a heterodimer with integrin β1, α5 (α5β1) orchestrates multiple stages of tumour cell dissemination in the bone, binding with fibronectin in the early stages of metastasis to provide essential adhesion sites for tumour cells [35, 45], and subsequently regulating tumour cell migration and invasion in the bone microenvironment [46, 47]. This evidence reflects the varied roles of integrins on tumour cell adhesion and migration to metastatic sites but does suggest mediated effects of a subset of integrins may be tumour-specific, as opposed to microenvironment-specific. Regardless, integrins remain a potential source of biomarkers for predicting or identifying bone metastasis prior to dormant cell awakening.

Inducing dormancy

MSCs and other cells from the osteoblastic lineage are thought to work within metastatic niches to induce tumour dormancy [48] (Fig. 2). BCCs co-cultured with MSCs exhibited down-regulation of the cancer stem cell marker CD44, resulting in significantly decreased proliferation and increased chemoresistance. Of note, whilst CD44 is used as a marker of cancer stem cells, it is not exclusively expressed by these cells [49]. BCCs in 3D co-culture with MSCs have shown a cancer cell 'cannibalism' effect, degrading and internalising MSCs. This resulted in enhanced cancer cell survival whilst also promoting a dormant phenotype through enriched tumour suppressors and pro-survival factors [50]. Tissue spatial heterogeneity has been suggested as one of the drivers for the failure of many anti-tumour therapies. Distinct tumour-niche interactions will occur in small compared to larger tumour lesions, or cells occupying the centre of a tumour compared to the outer, invasive front [45]. This can leave tumour cells at different stages of tumour dormancy, growth and progression. By considering the macroenvironment in addition to the tumour microenvironment, targeting both simultaneously, current therapeutic strategies could be improved [51, 52]. The advancement of spatial transcriptomics methods will significantly improve the knowledge in this field and begin to elucidate the importance of specific cell populations [53].

Recent findings have suggested a key role of exosomes in signalling between breast cancer cells and the metastatic environment. Incubation of exosomes collected from bone marrow-derived MSCs with cancer cells, promoted dormancy-related characteristics including inhibition of proliferation [49]. This is thought to be a result of microRNA (miR-222/223) delivery via exosomes that suppress the expression of a key proliferation-promoting protein [49, 58]. Interestingly, the content of secreted exosomes appears to change following contact with tumour cells, converting to 'primed' exosomes and further promoting a dormant phenotype in BCCs [59]. A novel therapeutic strategy targeting dormant BCCs has been proposed, utilising MSCs loaded with antagomiR-222/223, increasing dormant BCC sensitivity to chemotherapy and employing the 'awakening' strategy [2, 58]. Furthermore, the study of quiescent BCCs in the bone microenvironment implicated miR-127 and -197 in cell proliferation, transported from the bone marrow to tumour cells via gap junctions or exosomes, acting to reduce CXCL12 levels [60].

Factors secreted by the bone microenvironment, including bone morphogenic proteins (BMPs) can impact cell survival and growth, inducing tumour dormancy. BMPs and growth arrest-specific 6 (GAS6) produced by osteoclasts can directly inhibit DTC 205. Osteoblast-secreted factors, such as BMP7 and transforming growth factor β2 (TGF-β2) bind to DTC receptors and trigger the TGFβRIII– axis. This results in increased p38 activation and subsequent increased p38/ERK ratio, driving dormancy. Increased p38 levels results in increased activation of p53 and DEC2/SHARP1 (also known as BHLHB3) to induce quiescence, further contributing to tumour cell dormancy [61]. TGF-β2 and BMP7 from NG2+/Nestin+MSCs promote HSC quiescence in the BME activating a quiescent pathway via p38, inducing the activation of cell cycle inhibitor, p27 [62].

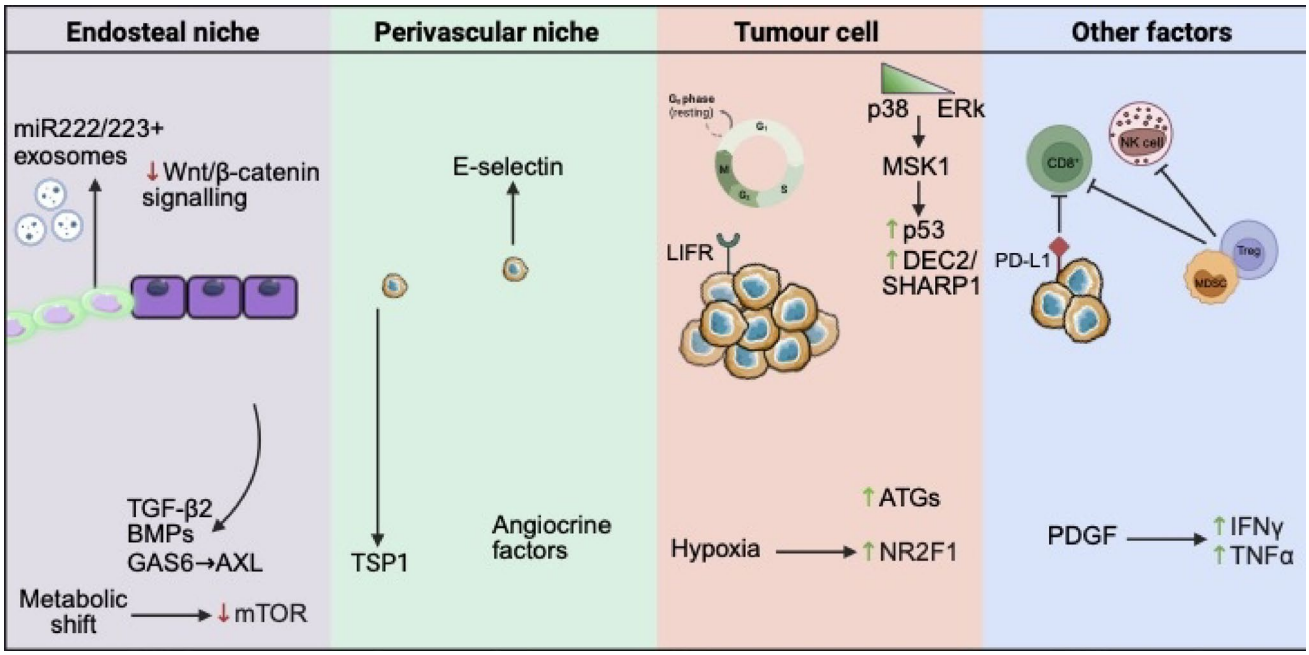

Fig. 2 The bone metastatic niches play distinct roles in promoting tumour dormancy, along with processes in the tumour cell itself. DTCs act in the endosteal niche to co-opt the HSC niche, promoting their survival in a dormant state, as well as encouraging MSCs to secrete miR-222/223 + exosomes, maintaining their dormant state. The release of TGFβ2 and BMPs from the bone surface can further promote a quiescent state. Angiogenesis suppressor, TSP1, promotes tumour dormancy from the perivascular niche. Tumour cell intrinsic processes that promote dormancy include a high p38/pErk ratio, increased MSK1 and p53 expression. Immune surveillance is down-regulated by PD-L1 and MDSC/Treg activity [5, 22, 23, 54–57]. Figure created with adapted images from Servier Medical Art (http://smart.servier.com) and BioRender (https://BioRender.com/nyigo7n), licensed under CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/)

Mitogen- and stress-activated protein kinase 1 (MSK1), a downstream effector of p38, has been shown to modulate dormancy by modulating the chromatin structure, reducing the expression of key genes required for luminal cell differentiation (GATA3 and FOXA1). In ER + breast cancer patients, low MSK1 is associated with early metastasis and downregulation in vivo impairs breast cancer cell differentiation and increases their bone homing capabilities [63]. The key osteoblast signalling pathway, Wnt/β-catenin, is implicated in the dormancy of cancer cells, yet the mechanisms remain unknown. Wnt5a, a Wnt signalling inhibitor from the osteoblastic niche, is downregulated in invasive breast carcinomas with higher histological grades, suggesting Wnt signalling acts to prevent tumour outgrowth [64]. Wnt5a acts via Seven In Absentia Homolog 2 (SIAH2) to repress Wnt/β-catenin signalling, dependent on the RTK-like orphan receptor (ROR2). Clinically, Wnt5a overexpression is observed in ER + breast cancers with a mutation of *PIK3CA*, seen in approximately 30% ER + breast cancers. All recurrent breast cancer cases in this study exhibited bone metastasis, highlighting the role of Wnt signalling in preventing the outgrowth of DTCs in bone [65].

Oxygen levels in bone typically lie between 2 and 9%, depending on the specific region. Hypoxia (1% O_2) in primary tumours has been shown to induce a dormant gene programme in DTCs and encourage nuclear receptor subfamily 2 group F member 1 (NR2F1) and hypoxia-inducible factor 1-alpha (HIF1α) to maintain the DTC stem cell state [66–68]. Acting together, NR2F1 and HIF1α maintain elevated expression of cell cycle inhibitor, p27, and down-regulation of cell cycle regulator, CDK4, sustaining DTCs in cell cycle arrest [66, 67]. Hypoxia-induced quiescence is more notable in ER + BCCs compared to TNBC. This is exemplified by ER + breast cancers being predominantly associated with bone metastasis and longer latency periods [69]. Small molecule CDK4/6 inhibitors, such as Palbociclib, are common first-line therapies for metastatic or advanced ER + / HER2− breast cancer, often given in conjunction with an endocrine therapy [70–72]. Whilst the inclusion of CDK4/6 inhibitors can improve the longevity of these therapeutics, emerging evidence suggests prolonged treatment can result in the proliferation of resistant subclones through multiple pathways, as reviewed by Glaviano et al. [73]. This is coupled with several off-target effects, including cardiotoxicity and highlights the need for a deeper mechanistic understanding of CDK functions [71, 72, 74].

In addition to low oxygen conditions, tumour cells need to endure limited nutrient supply to enter and remain in a quiescent state, requiring changes to metabolic pathways. Studies show DTCs have a decreased reliance on glycolysis

and instead shift to oxidative phosphorylation and fatty acid oxidation pathways. This metabolic shift involves mitochondrial biogenesis, increasing total mitochondrial mass, dependent on AMP-activated protein kinase (AMPK). Peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) acts alongside epigenetic regulation to alter metabolic gene expression and increase mitochondrial DNA [75]. Metabolic interactions between cancer cells and cells in the TME, including CAFs and immune cells, can influence factors such as nutrient availability and the metabolic signalling pathways utilised by dormant cells. Additionally, dormant tumour cells utilise autophagy to ensure proper energy balance, though this is thought to be an intrinsic process, and not microenvironment specific [76–78]. Under normal conditions, autophagy is regulated by the suppression of mammalian target of rapamycin (mTOR). During cellular stress or nutrient deprivation, mTOR kinase activity is suspended, preventing autophagy initiation and facilitating metabolite recycling and energy generation [77, 79].

Immune surveillance and inflammatory factors are key to tumour cell dormancy, both in inducing and promoting escape from dormancy. In tumour mass dormancy, proliferation is balanced by cell death due to immune surveillance from the immune system, utilising $CD4^+$ and $CD8^+$ T cells, interleukin (IL)-12 and interferon γ (IFNγ) production by NK cells and M1 macrophages [80]. Breast cancer cell secretion of platelet-derived growth factor D (PDGF-D) can be recognised by NK cells and promote the production of IFNγ and tumour necrosis factor (TNF)α, which act to induce dormancy [81]. Breast cancer cells induced into dormancy in this form exhibit enhanced stemness characteristics, suppressed STING signalling and upregulated BACH1/SOX2 expression, which collectively act to defend against NK cell attacks [82]. Dormant tumour cells will also express PD-L1 as part of the immune evasion efforts, allowing them to inhibit T-cell activity and avoid constant surveillance, maintaining their dormant state [83]. Bone marrow naturally has a high baseline of Tregs and myeloid-derived suppressor cells (MDSCs), resulting in reduced NK and T cell activities. Tumour cells exploit this immunosuppressive environment to remain dormant and avoid immune surveillance [54]. Secretion of TGF-β, vascular endothelial growth factor (VEGF) and indoleamine-2,3-dioxygenase (IDO), amongst other factors, by tumour and stromal cells can induce this MDSC-dependent immunosuppression, with IDO thought to be used by TNBC to reduce local levels of tryptophan, leading to production of cytotoxic metabolites [84].

Histone deacetylase (HDAC) inhibitors are implicated in breast cancer dormancy, acting to up-regulate leukaemia inhibitory factor receptor (LIFR) and promote a dormant phenotype [85–87]. Clinical trials investigating the effect of HDAC inhibitor treatment alongside standard of care chemotherapeutics are underway for advanced breast cancer [88], however early results are not promising, with HDAC inhibitors being reported to have no effects on progression free survival for patients with advanced breast cancer [89–91]. Subsequent pre-clinical studies have revealed an osteolytic effect of HDAC inhibitors in breast cancer bone metastasis models [85], suggesting that these compounds could stimulate release of bone bound growth factors, driving the vicious cycle of bone metastasis, thereby, negating any direct pro-dormancy effects on cancer cells in bone [86]. When combined with ZOL, HDAC inhibitor-driven bone loss was reduced, but not fully corrected, leaving the role of HDAC inhibitors unclear in breast cancer dormancy in bone [85, 92]. MALAT1, acting via Tead3, has also been implicated in the suppression of bone metastasis, impairing Nfatc1-drive osteoclast differentiation, inhibiting bone resorption, tumour awakening and metastasis [93]. However, as with many epigenetic changes in breast cancer bone metastasis, limited evidence exists of its role in dormancy [94].

Escaping dormancy

Tumour cells enter dormancy to enable survival until a time in which the conditions are favourable to proliferate. Once these conditions are met, mechanisms will begin to induce cell escape from dormancy (Fig. 3) [5, 18, 95]. Tumour dormancy is linked to osteoblast activity, whereas osteoclast-mediated mechanisms are thought to induce escape from dormancy and tumour growth. The recruitment of osteoclast precursors and increase in osteoclast activity, driven by vascular cell adhesion protein 1 (VCAM-1) has been shown to promote an escape from dormancy in dormant metastatic breast cancer cell lines [96]. Disruption of the canonical receptor activator of NF-κB/ligand (RANK/RANKL) interactions in osteoclasts with RANKL antagonist, osteoprotegerin-Fc (OPG-Fc), inhibits the growth of dormant DTCs *in vivo* [97]. Bisphosphonates have been widely used to improve symptoms of bone metastasis, reducing bone pain, skeletal fractures and hypercalcemia occurrence. They also have the potential to limit tumour reawakening and expansion, as seen with bisphosphonate treatment reducing in vivo breast cancer tumour growth in bone, despite the presence of DTCs remaining [98–100]. A meta-analysis of cancer risk and bisphosphonates revealed a significantly reduced risk of breast cancer with bisphosphonate treatment [101]. This is further supported by various clinical studies [102–107], including the AZURE trial, whereby adjuvant treatment of breast cancer patients with bisphosphonates

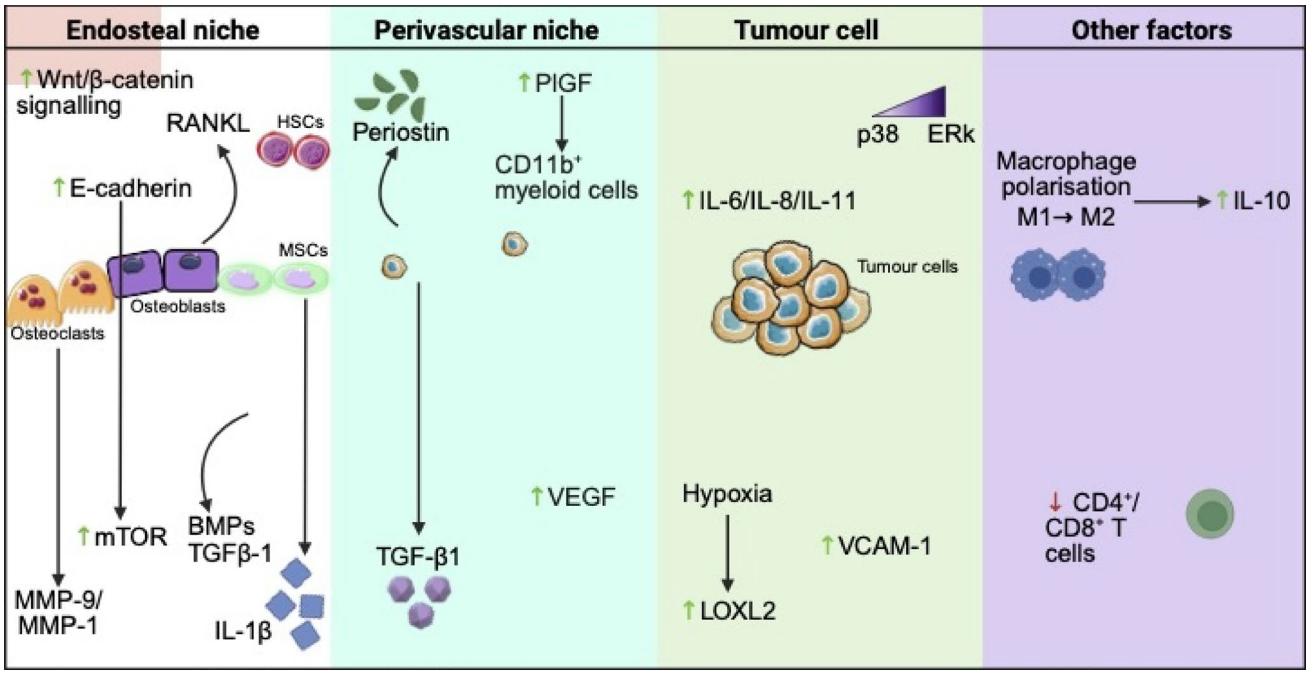

Fig. 3 The different bone metastatic niches will enact multiple effects on DTCs to promote an escape from dormancy. In the perivascular niche, angiogenesis promotes metastatic outgrowth by stimulating the release of periostin, with VEGF and PlGF acting alongside. The endosteal niche is responsible for the release of several factors thought to promote tumour micrometastasis, including TGFβ-1, BMPs and IL-1β. Tumour cells interact with osteoblasts to promote E-cadherin expression and increase mTOR activity, promoting tumour outgrowth. In the tumour cells, hypoxia can induce LOXL2 activity and as such, EMT, promoting invasive properties of tumour cells. This is coupled with a lower p38/pErk signalling ratio and increased VCAM-1 activity. Macrophage polarisation from M1 to M2 similarly promotes tumour reawakening via IL-10 [5, 22, 23, 55, 57, 109, 110]. Figure created with adapted images from Servier Medical Art (http://smart.servier.com) and BioRender (https://BioRender.com/ebe4wg0), licensed under CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/)

inhibits bone metastases and improves disease-free survival in post-menopausal patients [108].

RANKL-inhibitor Denosumab is similarly under investigation for the prevention of tumour cell reawakening in breast cancer bone metastasis and has shown superior responses in delaying skeletal-related events (SREs) compared to ZOL, in advanced breast cancer patients [103, 111–113]. However, a large cohort study in high-risk early breast cancer patients demonstrated no changes in disease-free survival following Denosumab therapy, compared to placebo [114], suggesting the mechanisms used by bisphosphonates to maintain dormancy in bone is not regulated by RANKL and its mechanism remains to be determined [111]. The effects of TGF-β on osteoblast, osteoclasts and bone remodelling are complex and are both spatial and temporal-dependent. While TGF-β2 signalling appears to have a pro-dormancy effect, TGF-β1 can promote bone metastasis through activation of specific genes, utilising the TGFβ-Smad signalling pathway. TGF-β2 signalling via Smad results in the production of several pro-osteolytic factors including IL-11, matrix metalloproteinase 1 (MMP-1), CXCR4 and parathyroid hormone related protein (PTHrP) [115, 116]. An animal model of breast cancer bone metastasis revealed the presence of active TGFβ-Smad signalling, specifically in the bone, with reduced bone metastases upon knockdown of Smad4 [117]. Coco, a secreted antagonist of TGF-β ligands, has recently been suggested as a key regulator of metastatic dormancy and reactivation of BCCs, blocking interaction of BMPs with their receptors. However, these effects are thought to be microenvironment specific, with Coco inducing a gene expression signature strongly associated with metastatic relapse to the lung, but not to the bone or brain [118].

Hypoxia has been discussed earlier in this review as a pro-dormancy microenvironmental trait [67, 68]. However, hypoxia can also be linked to an escape from dormancy. Conditional hypoxia induces lysyl oxidase-like 2 (LOXL2) expression in MCF-7 cells with dormant MCF-7 cells expressing LOXL2 having increased proliferation in the bone and exhibiting increased expression of the oestrogen receptor. Clinically, increased LOXL2 expression is associated with a decrease in relapse-free survival of breast cancer patients. Increased LOXL2 mRNA levels also correlated with increased EMT and stem cell markers, facilitating invasion of surrounding vasculature and hence, metastatic outgrowth [119]. HIF-1α mechanisms that have been determined to promote tumour dormancy can similarly be involved in tumour escape from dormancy and expansion, utilising VEGF production to promote angiogenesis and overcome

the oxygen shortage. HIF-1α can also impact immune surveillance to promote tumour growth by suppressing T-cells from killing tumour cells, facilitating further growth [120]. The duration of hypoxia is thought to impact whether dormancy is promoted, or tumour growth. However, the specific timeline that results in this shift is unclear [121, 122]. Mice exposed to acute cyclic hypoxia (12 cycles, 10 min on, 10 min off, 8% O_2) exhibited increased metastases [123], with intermittent hypoxia (6 cycles, 30 min on, 30 min off, 0%) similarly resulting in promoting an invasive breast cancer phenotype in vitro compared to chronic hypoxia (6 h, 0%) [124]. Of note however, is the varying time frames and conditions used for intermittent hypoxia experiments, ranging from 8 h 3x/week, to 24 cycles of 30 min normoxia/hypoxia, utilising hypoxic oxygen concentrations of 0.1% to 2%, yet all resulting in increased breast cancer progression phenotype [124]. Conversely, prolonged hypoxia (24–48 h, 0.5% O_2) diminishes LIFR, increasing IL-6 expression and downregulation of dormancy-, quiescence- and cancer stem cell-associated genes [87, 125]. Similarly, cancer cells incubated under chronic hypoxia conditions (16–48 h, 0–0.02% O_2) showed a more invasive phenotype, yet it is suggested that chronic hypoxia can also render tumour cells incapable of replication following reoxygenation, preventing tumour outgrowth [126]. A key theme throughout studies of hypoxia in tumour dormancy is the variability in conditions used and the labels applied to these e.g. acute vs chronic, limiting the ability to draw a definitive conclusion [124, 127].

MMPs are a family of enzymes that play a key role in the breakdown and remodelling of the ECM and have been implicated in tumour dormancy. MMP-9 is highly expressed by osteoclasts and utilised for osteoclast migration and resorption. High levels of MMP-9 are observed in BCCs, acting in an integrin-αvβ3-dependent manner to promote the migration of MDA-MB-435 cells from bone metastatic sites in mice [128]. MMP13 is also linked to tumour cell awakening, with inhibitor (5-(4-{4-[4-(4-fluorophenyl)-1,3-oxazol-2-yl]phenoxy}phenoxy)-5-(2-methoxyethyl) pyrimidine-2,4,6(1H,3H,5H)-trione (Cmpd-1)) preventing primary tumour growth and reducing the onset of osteolytic lesions following intra-cardiac and intra-mammary injections of MDA-MB-231 cells in mouse models. No effects were observed in soft organ metastases, suggesting MMP13 regulation of dormancy maybe specific the bone microenvironment, and highlighting a potential therapeutic target of interest [129].

Epigenetic regulation of tumour cells plays a role throughout the stages of dormancy. Biopsies from bone metastatic breast cancer patients revealed promotor methylation and reduced expression of High in Normal 1 (*HIN-1*), Retinoic Acid Receptor Beta (*RAR-β*), and Ras Association Domain Family Member 1A (*RASSF1A*) [130], compared to disease-free tissue, all linked to cancer cell plasticity and EMT [130, 131]. This suggests hypermethylation of these tumour suppressors could act as a potential biomarker for bone metastatic progression, improving detection and therapeutic options. Enhancer of zest homolog 2 (EZH2), acting via H3K27me3, also causes changes in gene transcription and epigenetic reprogramming of breast cancer cells in the bone, enhancing their metastatic spread from bone. EZH2 inhibition and knockdown in vivo resulted in impaired metastasis to secondary organs, yet this was after bone lesions were already observed [132]. This highlights the epigenome as a dynamic regulator of tumour cell behaviour but also suggests its primary role may be the regulation of metastasis from the bone, rather than the re-awakening of dormant cells in the bone [94].

Interactions between tumour cells and surrounding bone, stromal and endothelial cells plays an important role in mediating the angiogenic switch. Bone-marrow derived endothelial cells produce high amounts pro-angiogenic factors, including Id-1. Reduced production of these factors significantly reduces the formation of micrometastases in mouse breast cancer models [55, 133]. The release of VEGF from the bone environment promotes tumour angiogenesis, increases oxygen and nutrient delivery, and allows for tumour growth [55, 134, 135]. VEGF action has also been linked to promoting escape from tumour dormancy in diet-induced obesity (DIO) mouse models. VEGF is upregulated alongside lipocalin-2 (LCN2) and basic fibroblast growth factor (bFGF) with increased tumour frequency and reduced tumour latency observed, bridging the link between obesity and the severity of post-menopausal breast cancer [7]. Placenta growth factor (PlGF) production by BCCs triggers the recruitment of $CD11b^+$ myeloid cells from the bone marrow and increases angiogenesis. Breast cancer patients with increased plasma PlGF levels exhibit increased metastasis and a poorer prognosis, implicating PlGF in the outgrowth of breast cancer cells in bone metastasis by overcoming angiogenic dormancy [136].

The bones natural immunosuppressive nature aids in tumour cell evasion of immune clearing. However, when tumour cells are awakening from dormancy, the release of immune suppressive cytokines triggers macrophage polarisation, from M1 to M2, also known as tumour-associated macrophages (TAMs) [109, 110]. M2 macrophages are characterised by increased production of IL-10 and TGF-β, altering the activation of $CD4^+$ and $CD8^+$ T cells [137, 138]. Other interleukins are also implicated in tumour growth in bone. IL-8 and IL-11 release from breast cancer cells has been shown to increase osteoblastic RANKL production and mediate direct effects on promoting bone metastasis *in vivo* [117, 139]. IL-1β has been strongly linked to the reawakening of dormant DTCs via activation of Wnt signalling in the

tumour cells, regulating changes to the bone microenvironment [140]. In BCCs, this action of IL-1β has been linked to the demethylating actions of ten-eleven translocation proteins (TETs), whose inhibition results in reduced EMT and bone metastasis markers [141]. Inhibition of IL-1β signalling in mouse models results in decreased osteoblast and osteoclast activity and inhibition of metastatic outgrowth of breast cancer cells in bone, whilst also exhibiting effects on anti-tumour immune cells in an innate response [142, 143]. Conversely, tumour cells expressing high levels of IL-1β increase osteoclast activity, and metastatic outgrowth in mouse bone [144, 145]. From a clinical perspective, higher expression of IL-1β in primary tumours is associated with bone metastasis in breast cancer patients (37% cases with IL-1β vs 5% cases without). Interestingly, this link is independent of ER receptor status in tumours suggesting that IL-1β may be a useful biomarker for predicting future relapse in bone [144, 146]. Additionally, inhibition of IL-1β or it's receptor IL-1R have been proposed as potential treatment strategies to prevent IL-1β-mediated escape from dormancy [2, 147]. By stimulating bone resorption, these factors allow for the release of pro-tumour growth factors, promoting tumour reawakening.

Tumour progression

As discussed, increased osteoclast activity is associated with an escape from dormancy and progression to metastasis. In bone specifically, DTCs release PTHrP, inducing osteoblast-mediated up-regulation of RANKL and down-regulation of OPG [67, 97, 148]. Increased TGF-β release from resorbed bone enables cancer cell proliferation and acts to further increase PTHrP from tumour cells via the activation of SMAD and p38 MAPK pathways, stimulating further osteoclast differentiation, bone resorption and TGFβ release [149]. Clinically, PTHrP expression increases from ~ 60% in primary tumours to 92% in cases of breast cancer bone metastasis, compared to 17% in tumours metastasised to other sites [148, 150–152]. TGFβ also promotes the expression of tumour-growth-promoting cytokine, IL-6, from stromal cells and osteoblasts via Jagged1, further promoting the cancer cell proliferation [23, 153]. This process results in a self-sustaining positive-feedback loop called the 'vicious cycle' (Fig. 4) [1, 2, 4, 154]. Further contributions to the 'vicious cycle' are observed by integrin α5β1, through modulation of bone turnover. Tumour cells exploit α5β1-signalling to secrete TGFβ and RANKL, disrupting the bone remodelling balance [44, 155]. Circular RNA, circIKBKB, has also been shown to upregulate RANKL and PTHrP, promoting breast cancer metastasis and tumour awakening [156]. Targeting circIKBKB and related signalling components could present a novel therapeutic strategy for halting the vicious cycle and preventing tumour progression in the bone. A key factor with tumour dormancy is the potential for residual disease to re-enter dormancy. There is currently no cure for bone metastasis, and while therapies may reduce the tumour burden on the bone, the risk of further dormant cells reactivating is ever-present, further complicating therapeutic efforts.

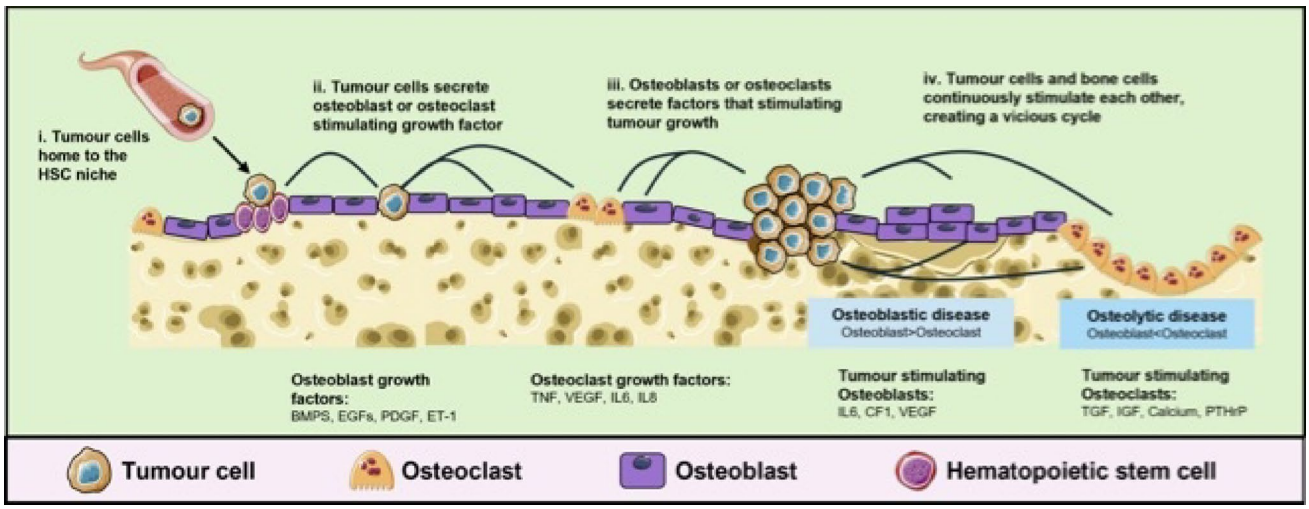

Fig. 4 The bone marrow is a common site of tumour metastasis with the microenvironment being a major contributor to tumour growth characteristics. Tumour cells will home to the HSC niche in the bone microenvironment and secrete growth factors to promote osteoblast and osteoclast activity. Increased bone cell activity will result in increased secretion of tumour growth-promoting factors, creating a positive feedback loop, termed the vicious cycle [1, 2, 4, 23]

Methods to study bone metastasis

The extent of mechanisms involved in tumour dormancy and metastasis in the bone exemplifies the complexity of developing therapeutics. This is further complicated by the limitations of models available to reflect dormancy (Table 1). Many techniques have been developed for cancer research or bone studies but not yet applied to dormancy. In vitro models are commonly used in more preliminary stages for cancer studies to understand cell to cell signalling, impacts on gene expression or signalling pathways. Several 3D tumour cell/bone cell co-culture models of dormancy have been generated over time to improve these early experiments [55, 157–161]. However, these models lack the presence of mineralised bone, ECM components and other cell types, such as endothelial, stromal or immune cells, lacking observation of these interactions. An advancement on cell line 3D cultures is the development of patient-derived organoid (PDO) models, whereby organoids maintain their in vivo genetic and phenotypic heterogeneity, as well as patient-specific drug sensitivities, but these have not yet been incorporated into bone dormancy research [162, 163].

Organ-on-a-chip solutions are being advanced in many fields, including bone [69, 171–173]. These systems are

Table 1 Experimental methods for the study of bone metastasis and tumour dormancy

	Technique	System Type	Output	Strengths	Limitations
In vitro	2D In Vitro Co-culture Systems [17, 55, 87, 164–166]	Monolayer co-culture of tumour cells with bone marrow cells	Cell-to-cell interactions, dormancy-associated signalling and gene expression (e.g., p38/ERK ratio)	Highly controlled; possibility for genetic manipulation; cost-effective	Lacks bone microenvironment context and 3D architecture
	3D In Vitro Models (Spheroids, Organoids, Scaffold-based Systems) [55, 157–161, 167–170]	Tumour spheroids embedded in scaffolds formed from synthetic polymers, natural fibres or hydrogels, co-cultured with bone marrow cells	ECM stiffness; integrin signalling; hypoxia	3D architecture; reflects mechanical cues; better mimics quiescent phenotype; adjustable stiffness	Limited immune and vascular components; simplified niche
	Bone-on-a-Chip / Microfluidic Platforms [171–177]	Engineered microfluidic bone-mimetic systems	Perivascular niche dynamics	Reproducible; real-time monitoring; adjustable microenvironment; cost-effective (compared to in vivo)	Technical complexity; limited long-term modelling; expensive start up costs
Ex vivo	Ex Vivo Bone Explants [178–181]	Murine or human bone fragments cultured with tumour cells	Native bone matrix with full trabecular structure	Preserves physiological ECM; retains bone-resident cells	Short culture lifespan; limited immune components
In vivo	Syngeneic Mouse Models [87, 135, 182, 183]	Murine tumour cells in mice	Immune-mediated dormancy and escape	Intact immune system; complete niches present	Species-specific tumour biology; fewer bone-specific models
	Xenograft Mouse Models [63, 87, 97, 100, 182]	Human breast cancer cells in mice	Bone invasion and metastatic growth	Human tumour biology; established protocols; longitudinal monitoring ()	May favour overt metastasis over dormancy; species-specific bone biology
	Patient-Derived Xenografts (PDX) [167, 184]	Primary patient tumour implanted into immunodeficient mice	Preserves tumour heterogeneity; clinically relevant dormancy traits	Highly translational; maintains genomic complexity	Expensive; low throughput; immune-deficient host; species-specific bone biology
	Intravital Imaging (IVI) [185–189]	Live imaging in bone windows	Real-time DTC behaviour; cell–niche interactions	Direct visualisation of dormancy; spatial resolution	Surgical expertise required; limited field of view; limited scope to perivascular niche
In silico	Computational modelling [190–192]	Integration of biological findings with computational measurements and calculations	Molecular interactions, cell-niche interactions	Reproducible; complete niche inclusion; adjustable parameters	Inability to process native physiology; complex analysis
Downstream Analysis	Single-Cell RNA Sequencing (scRNA-seq) [87, 132, 193]	Transcriptomic profiling of isolated DTCs and niche cells	Cellular heterogeneity; dormancy gene signatures	High-resolution molecular profiling; complete pathway discovery	Lack of spatial context; limited analysis of rare cells; expensive; complex analysis
	Spatial Transcriptomics [42, 53, 193]	Spatial gene expression in bone sections	Localisation of DTCs within niches; tissue spatial heterogeneity	Maintains spatial context; niche mapping	Lower single-cell resolution (platform-dependent); expensive; limited standardised analysis pipelines

advanced in modelling the perivascular niche compared to other in vitro or ex vivo methods. However, they are limited by their timeline, typically lasting for 2–4 weeks, preventing a true understanding of clinical dormancy timescales [171, 172]. Conversely, in vivo models can better reflect these timescales. Breast cancer dormancy in bone has been successfully exhibited in xenograft mice following intra-cardiac injection of human tumour cells [97, 100]. Xenografted mice can often succumb to the results of primary tumours before metastasis and dormancy occurs As such, models have been developed to transplant cells into the orthotopic site and surgically remove tumours once they reach a certain size limit, allowing for subsequent metastasis development and monitoring [194, 195]. Patient-derived xenografts (PDX) improve further compared to the use of tumour cell lines in vivo, better reflecting clinical dormancy traits, but are still subject to species-specific differences [167, 168, 184].

With tissue spatial heterogeneity growing as an area of interest, the impact of spatial transcriptomics is similarly growing [42, 53, 193]. Recent advances have allowed spatial transcriptomics approaches to be applied to the study of human bone [169], but not yet dormancy, with current data sets lacking sufficient sample numbers for conclusive interpretation [53, 169, 193]. As with all experimental setups, it is important to utilise a range of models to draw informed conclusions about DTC behaviour in bone, further helping with the effective delivery of therapeutics.

Therapeutic approaches for tumour dormancy

Development of effective interventions to target dormant, as well as proliferative, tumour cells is essential to prevent relapse and improve cancer survival rates. Theorietically this could be achieved by targeting any of the four distinct stages of metastatic progression: dissemination, dormancy, reactivation or tumour growth (Fig. 5). Bone colonisation of tumour cells could be prevented by utilising anti-adhesion strategies such as CXCR4 inhibitors. However, DTCs can be detected in > 60% of patients with early breast cancer [10–16], suggesting dissemination occurs extremely early on in tumour development, limiting the value of this therapeutic strategy. Efforts are being made to maintain tumour dormancy in patients, or prevent escape mechanisms, often referred to as the 'sleeping' strategy [196, 197], to prevent proliferative growth and metastasis using drugs such as HDAC inhibitors [85–87], or NR2F1 agonists [66–68, 198]. While this presents promise, a reservoir of disease remains in the patient, presenting a significant risk of dormant cell awakening through alternative mechanisms not being targeted. Furthermore, clonal heterogeneity and therapeutic resistance are established drivers of treatment failures, as seen with CDK4/6 inhibitors [73].

Suppressing metastasis appears to be one of the most promising approaches, using the 'awakening' strategy, re-awakening dormant tumour cells, sensitising them to chemotherapy-based treatments, or the 'killing' strategy, eliminating dormant tumour cells whilst in their quiescent state [196, 197]. However, many current methods such as chemotherapies and combined bone-targeting approaches are largely focused on reducing tumour burden once progression has begun, addressing only part of the problem. Eliminating dormant tumour cells whilst in their quiescence state would provide a huge advancement in therapeutics, yet this is still limited by difficulties in the detection of dormant cells, as effective treatment would be difficult to confirm. The use of nanotechnology delivery systems is of current interest and is being investigated for several solid tumours [199, 200]. This targeting strategy may improve current efforts to reach lesions and DTCs buried deep within the bone [201–203].

Conclusion

The complicated interplay between cancer cells and the bone microenvironment provides a daunting barrier to long-term survival for patients with breast cancer bone metastasis. This review details a wide range of mechanisms used by tumour cells to metastasise and the impact of different pathways on tumour dormancy. It is clear from pre-clinical evidence that distinct mechanisms are used between cancer types and metastatic sites, removing the possibility of a 'one-size fits all' approach. Several major questions remain. What markers or landmarks can be used to identify dormant tumour cells, as opposed to slow-cycling or therapy-resistant cells? How does spatial heterogeneity and tissue cell distribution impact dormant tumour cell behaviour and the timeline of their awakening? What, if any, combination of triggers is required to promote escape from dormancy and what drives the variability between these latency periods?

Moving forward, the field must transition from identifying general pathways to determining the best approaches to target these pathways for therapeutic development. The development of biomarkers for dormant tumour cells in bone is essential in detecting dormant cells before progression, enabling early intervention. In parallel, refining a 'sleeping strategy' approach to therapeutics remains a highly promising path toward improving long-term outcomes for patients with breast cancer bone metastasis. Leveraging current technological advances in modelling tumour cell dormancy

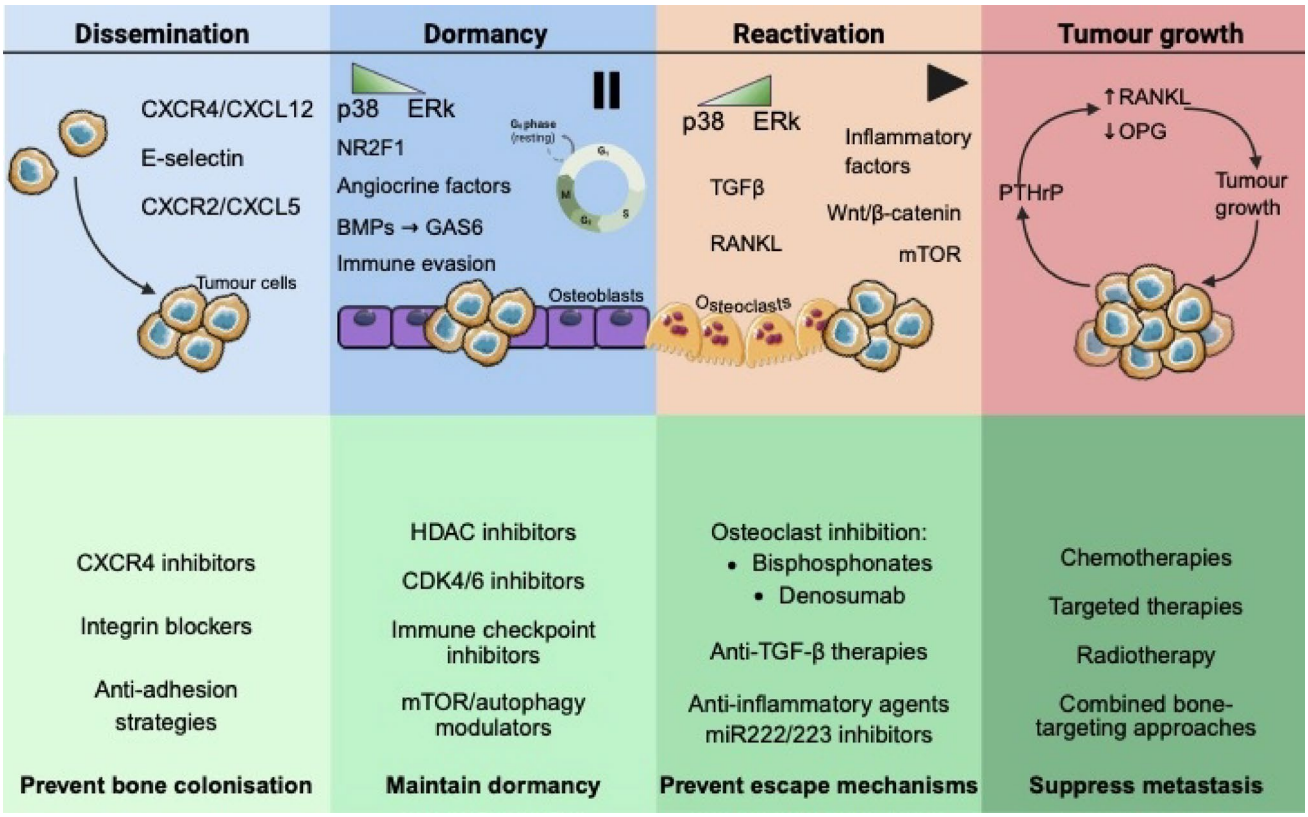

Fig. 5 Schematic representation of breast cancer dissemination, dormancy, awakening and progression in the bone. Therapeutic strategies can target different time-points of tumour dormancy in bone, utilising the 'sleeping', 'awakening' and 'killing' strategies. This highlights the range of mechanisms currently under investigation, with a combination approach potentially required to target different stages of dormancy [2, 74, 83, 85, 94, 99, 111, 112, 112]. Figure created with adapted images from Servier Medical Art (http://smart.servier.com) and BioRender (https://BioRender.com/ebe4wg0), licensed under CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/)

in bone is key to the success of further studies and therapeutic approaches.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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