BONE METASTASES

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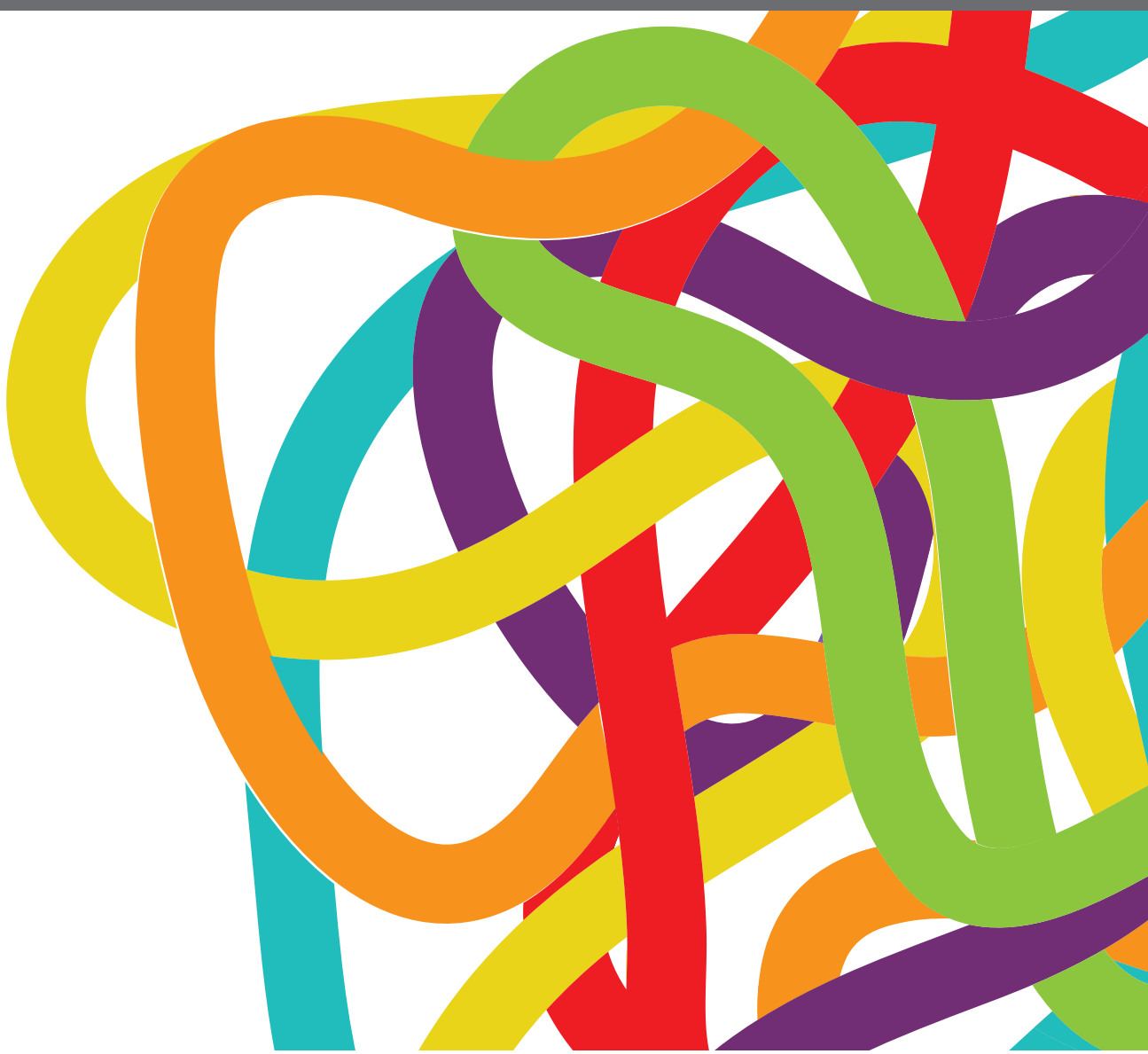

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BONE METASTASES

Topic Editors:
Maria Teresa Valenti, University of Verona, Italy
Olivier Feron, Université catholique de Louvain, Belgium

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EDITORIAL
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Editorial: Bone Metastases

Maria Teresa Valenti[1]*, *Monica Mottes*[2], *Luca Dalle Carbonare*[1] and *Olivier Feron*[3]

[1] Department of Medicine, University of Verona and Azienda Ospedaliera Universitaria Integrata Verona, Verona, Italy, [2] Department of Neurosciences, Biomedicine and Movement Sciences, University of Verona, Verona, Italy, [3] Pole of Pharmacology and Therapeutics (FATH), Institut de Recherche Expérimentale et Clinique (IREC), UCLouvain, Brussels, Belgium

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Editorial on the Research Topic

Bone Metastases

Solid tumors often metastasize into bones affecting quality of life and overall survival of cancer patients. Despite improvement of diagnostic tools, the presence of bone metastases often reveals an advanced disease stage with a median survival of a few months and limited appropriate therapies. Moreover, patients with bone metastases suffer from considerable morbidity including pain, fractures and hypercalcemia (1). Bone metastases are often detected in patients with breast, prostate and lung cancers but it is also increasingly recognized that the ability of other types of cancer to form bone lesions has been underestimated for many decades.

Metastasization is a multi-step process where cancer cells escape from the primary tumor, intravasate, survive in the bloodstream and later extravasate from the circulation to develop at a distant site; lymphatics represent another route for migrating cancer cells that will first colonize nodes and later on potentially reach the circulation. Adaptation to the new environment is a pre-requisite for effective growth in conditions different from the primary site. Bone metastases result from complex interactions of cancer cells with hematopoietic stem cells, endothelial cells as well as bone cells (osteoblasts with bone forming activity and osteoclasts with bone resorption activity). In the last years, the contribution of osteoclasts to bone metastasization has been largely investigated including through the dissection of their crosstalk with cancer cells (2). The latter were for instance documented to play a central role in destroying bone upon lowering of extracellular pH, a prerequisite for osteoclast activity (3, 4). Other studies have revealed that cancer cells interact with bone cells either to modulate their dormancy or promote drug resistance (5, 6).

This Research Topic includes two reviews that paint a very broad picture of how the interplay between tumor and bone-resident cells drives the local development of metastases. While Gyori and Moscai review the different osteoclast signaling pathways that are related to the pathological bone loss, Haider et al. describe the interaction between tumor cells and endosteal niche cells during the early stages of breast cancer bone metastasis, with a particular focus on mesenchymal-derived osteoblasts and fibroblasts. The role of calcium, an important building block of bones but also a recognized actor in the development of bone metastases is also addressed in this RT. Through the review of major calcium channels and/or calcium-related routes (ie, TRPs, VGCCs, SOCE, and P2Xs), Yang et al. provide evidence that alterations in calcium homeostasis in bone metastases directly participate in tumor progression. Das et al. also examine the role of calcium by focusing on the Ca^{2+}-sensing receptor (CaSR), a dimeric class-C G protein-coupled receptor (GPCR). In their review, these authors explore the hypothesis of CaSR acting as an oncogene in breast cancer and

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Tao Liu,
University of New South Wales, Australia

***Correspondence:**
Maria Teresa Valenti
mariateresa.valenti@univr.it

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associated bone metastases, facilitating a vicious cycle wherein osteolysis promotes tumor growth and inversely.

Several articles within this RT explore recent developments in the search for therapeutic strategies targeting bone metastases. Kratzsch et al. describe how mTOR inhibitor everolimus but also axitinib, a specific VEGF receptor tyrosine kinase inhibitor, may prevent and retard formation of symptomatic spinal metastases. Interestingly, La Manna et al. who used patient-derived organoids and xenografts (PDX) models from bone-metastatic prostate cancer report the therapeutic benefit of mTORC1/2 inhibitor Rapalink-1, further supporting a role of mTOR pathway in cancers metastasizing in bones. Besides the above anti-oncogenic approaches, targeting metabolism represents another option which may take advantage of the emerging development of new drugs in this field. Tiedemann et al. examine current evidence underlying how altered metabolism in cancer cells may impact on substrate availability for bone cells, and on consecutive alterations in osteoclast differentiation and activity. Xu et al. follow a more focused approach examining how a component of the electron transport chain complex I (ie, NDUFA4L2) accounts for epithelial-to-mesenchymal transition of osteosarcoma cells, pointing out OXPHOS as a critical metabolic path in the development of primary malignant bone tumors. Finally, Xu et al. discuss recent progress on evaluating the role of endoplasmic reticulum stress on bone metastases, identifying a set of potential targets to develop new therapeutic modalities directed against bone metastases. Besides therapeutic approaches, this RT also emphasizes the obvious interest to identify patients at risk for bone metastases in order to treat them at an earlier stage and thereby improve clinical outcomes; current studies evaluating such prognostic biomarkers are summarized by Iuliani et al.

Altogether, the different contributions to this RT offer a series of insightful sets of data to better understand mechanisms involved in the multi-cellular process driving bone metastases and open new perspectives of treatment to counteract the onset of this major life-threatening cancer complication.

AUTHOR CONTRIBUTIONS

MV, MM and LD prepared the draft. OF supervised and wrote the paper. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Coleman RE, Croucher PI, Padhani AR, Clezardin P, Chow E, Fallon M, et al. Bone Metastases. *Nat Rev Dis Primers* (2020) 6:83. doi: 10.1038/s41572-020-00216-3
2. Hiraga TJ. Bone Metastasis: Interaction Between Cancer Cells and Bone Microenvironment. *J Oral Biosci* (2019) 61:95–8. doi: 10.1016/j.job.2019.02.002
3. Yuan F-L, Xu M-H, Li X, Xinlong H, Fang W, Dong JJ. The Roles of Acidosis in Osteoclast Biology. *Front Physiol* (2016) 7:222. doi: 10.3389/fphys.2016.00222
4. Corbet C, Feron OJ. Tumour Acidosis: From the Passenger to the Driver's Seat. *Nat Rev Cancer* (2017) 17:577. doi: 10.1038/nrc.2017.77
5. Croucher PI, Mcdonald MM, Martin TJJ. Bone Metastasis: The Importance of the Neighbourhood. *Nat Rev Cancer* (2016) 16:373. doi: 10.1038/nrc.2016.44
6. Kolb AD, Shupp AB, Mukhopadhyay D, Marini FC, Bussard KMJ. Osteoblasts Are "Educated" by Crosstalk With Metastatic Breast Cancer Cells in the Bone Tumor Microenvironment. *Breast Cancer Res* (2019) 21:1–30. doi: 10.1186/s13058-019-1117-0

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REVIEW
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The CaSR in Pathogenesis of Breast Cancer: A New Target for Early Stage Bone Metastases

Souvik Das[1]*, *Philippe Clézardin*[2,3], *Said Kamel*[1,4,5], *Michel Brazier*[1,4,5] and *Romuald Mentaverri*[1,4,5]

[1] MP3CV, EA7517, CURS, University of Picardie Jules Verne, Amiens, France, [2] INSERM, Research Unit UMR_S1033, LyOS, Faculty of Medicine Lyon-Est, University of Lyon 1, Lyon, France, [3] Department of Oncology and Metabolism, Medical School, University of Sheffield, Sheffield, United Kingdom, [4] Department of Biochemistry, Amiens-Picardie University Hospital, Amiens, France, [5] Faculty of Pharmacy, University of Picardie Jules Verne, Amiens, France

The Ca^{2+}-sensing receptor (CaSR) is a class-C G protein-coupled receptor which plays a pivotal role in calciotropic processes, primarily in regulating parathyroid hormone secretion to maintain systemic calcium homeostasis. Among its non-calciotropic roles, where the CaSR sits at the intersection of myriad processes, it has steadily garnered attention as an oncogene or tumor suppressor in different organs. In maternal breast tissues the CaSR promotes lactation but in breast cancer it acts as an oncoprotein and has been shown to drive the pathogenesis of skeletal metastases from breast cancer. Even though research has made great strides in treating primary breast cancer, there is an unmet need when it comes to treatment of metastatic breast cancer. This review focuses on how the CaSR leads to the pathogenesis of breast cancer by contrasting its role in healthy tissues and tumorigenesis, and by drawing brief parallels with the tissues where it has been implicated as an oncogene. A class of compounds called calcilytics, which are CaSR antagonists, have also been surveyed in the instances where they have been used to target the receptor in cancerous tissues and constitute a proof of principle for repurposing them. Current clinical therapies for treating bone metastases from breast cancer are limited to targeting osteoclasts and a deeper understanding of the CaSR signaling nexus in this context can bolster them or lead to novel therapeutic interventions.

Keywords: CaSR, calcium-sensing receptor, breast cancer, mammary gland, bone metastasis, calcilytics

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Maria Teresa Valenti, University of Verona, Italy

Reviewed by:
Svetlana Komarova, McGill University, Canada
Thierry Capiod, Institut National de la Santé et de la Recherche Médicale (INSERM), France

***Correspondence:**
Souvik Das
souvik.das@u-picardie.fr; souvik.das710@gmail.com

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INTRODUCTION: THE CALCIUM SENSING RECEPTOR

The concept that extracellular Ca^{2+} acts directly on parathyroid cells to regulate PTH secretion had been afloat since the 1960's (1, 2). However, the first precise assertion of the hypothesis suggesting a "possible existence of a divalent cation receptor" on the cellular surface came from electrophysiological experiments performed in rat parathyroid cells in 1983 (3). The concept of a calcium receptor gained traction in the 1980's, and by 1990 several characteristics had been revealed. In 1991, two independent groups (4, 5) showed a viable strategy for cloning the calcium receptor in X*enopus* oocytes, an approach later used by Ed Brown et al. in cloning the cDNA encoding the bovine parathyroid calcium receptor (6). The irrefutable evidence on the existence of the receptor in 1993 was further reinforced by the clinically significant discovery that mutations in the calcium sensing receptor gene gave rise to inherited disorders of disrupted calcium homeostasis (7).

The extracellular CaSR is a dimeric class-C G protein-coupled receptor (GPCR), closely related to metabotropic glutamate receptors, gamma-aminobutyric acid type B ($GABA_B$) receptors, various taste receptors and pheromone receptors. The human CaSR is a 1,078 amino acid protein, with a large 612 amino acid extracellular domain making up two lobes which adopt a Venus flytrap (VFT) conformation (8). Upon agonist stimulation, an open cleft of the VFT closes in, which is believed to induce conformational changes in the other domains, initiating signal transduction (9). Although the nomenclature points toward the main ligand of this receptor (Ca^{2+} ion), it does little to disclose its promiscuity of responding to various di- and trivalent cations, basic polypeptides, amyloid β-peptides and some aminoglycoside antibiotics (10–14). These constitute orthosteric agonists or type I calcimimetics which stimulate the receptor in the absence of Ca^{2+} or increases the sensitivity to calcium, albeit with different potencies. The second type of CaSR agonists are called allosteric modulators. These generally bind to a site different from that of orthosteric agonists, affecting the signaling and affinity of the orthosteric agonists either positively (calcimimetics) or negatively (calcilytics).

Signaling through the CaSR is multifaceted. Based on the majority of studies of this receptor in parathyroids, it has been shown to mainly interact with Gq/G11 heterotrimeric G protein (15, 16). Various intracellular cascades finally lead to a decrease in the secretion of parathyroid hormone (PTH) and a reduction in renal tubular Ca^{2+} reabsorption (17). Intracellular Ca^{2+} kinetics has been reported to be influenced by G12/13 pathways in different cell types. An example of such a modulation has been reported in the bone, where a G12/13 mediated activation promoted osteoblastic differentiation and downregulated osteoclastogenesis (18, 19). Also, since G12/13 signaling has been implicated in cell migration, it has been hypothesized to aid metastatic spread of breast and prostate tumors (20–22). CaSR mediated Gs signaling has been observed in pituitary cells and has been shown to affect human fetal lung development (23, 24). Being a multimodal chemosensor involved in transducing extracellular metabolic signals, the CaSR is also involved in preferential activation of distinct intracellular pathways in a phenomenon termed as "biased signaling" or "stimulus bias" (25). This is being leveraged in contemporary strategies for drugs targeting GPCRs (including the CaSR) while minimizing side-effects (26, 27). The alternation in coupling of G-proteins between normal and transformed breast cells was first hypothesized by Mamillapalli et al. and we have summarized it separately in our review as this is an important facet. This section aims to provide an opportunity to appreciate the various evidences of multiple G-protein couplings of this GPCR without deep-diving into the details of the downstream signaling pathways. For a comprehensive discourse on signaling, one can refer to an excellent review by Conigrave et al. (25).

The CaSR senses minor perturbations in serum Ca^{2+} levels and thus maintains an equilibrium by tightly regulating PTH secretion, renal calcium control, and bone remodeling. When the CaSR senses a dip in the extracellular Ca^{2+} concentration, it induces PTH secretion from the parathyroid glands. The secreted PTH acts by reducing kidney Ca^{2+} excretion, increasing intestinal Ca^{2+} absorption, and increasing bone resorption to release skeletal Ca^{2+}. On the other hand, an increase in the physiological Ca^{2+} level causes receptor activation and inhibition in PTH synthesis and secretion (28). As already mentioned, the physiological significance became apparent when various inherited disorders like familial hypocalciuric hypercalcemia (FHH) and neonatal severe hyperparathyroidism (NSHPT) were found to be caused by loss-of-function mutations in the CaSR gene (7). Alternatively, gain-of-function mutations were found to give rise to autosomal dominant hypocalcemia (ADH) and Bartter Syndrome type V (29, 30). However, the receptor is not restricted to calcium homeostasis but also has been implicated in diverse processes like cellular proliferation, cellular differentiation, secretion, and gene expression in different tissues such as stomach, intestines, skin, brain, liver, and heart (31–39). Before delving into a rationale for targeting this versatile receptor in breast cancer, we will briefly look into its function in the mammary gland and how it plays a role in cancer progression.

CASR IN THE NORMAL MAMMARY FUNCTION

The first report of localization and confirmation of expression of CaSR in human breast tissues was published by Dr. Edward M. Brown's laboratory (40). They observed the expression of both mRNA and protein levels in ductal epithelial cells which was further confirmed by successive findings in mice (41). Immunofluorescence staining of lactating glands revealed the expression of the receptor in luminal epithelial cells at the basolateral surface and in the cytoplasm (41, 42). Although it is reasonable to surmise that the CaSR is mainly located on epithelial cells in the breast, these studies do not exclude minimal presence of the receptor in stromal cells (43). The role of the receptor was elucidated to be involved in controlling lactation by modulating the production of PTHrP and regulating calcium (41). The expression of CaSR in mammary epithelial cells was subsequently shown to be upregulated during lactation and downregulated during pregnancy (41). To circumvent neonatal deaths from a homozygous CaSR gene disruption, the CaSR gene was disrupted in mammary epithelial cells in mice at the onset of lactation which resulted in altered maternal calcium homeostasis (44). This genetic ablation had a domino effect- the lactating mothers were hypercalcemic, showed decreased PTH secretion (with increased renal excretion of calcium), increased secretion of PTHrP into milk, and reduced calcium transport into the milk (44). The suckling neonates demonstrated decreased calcium accumulation, evident from their ash calcium content (44). Although the lactating breast coordinates maternal and neonatal calcium homeostasis, it can be concluded from studies till date that the CaSR does not play a pivotal role in the development or differentiation in the normal mammary gland. The caveat of this conclusion is that most studies have focused on the period of lactation where there is the highest expression of CaSR (42).

Culmination of intensive work at understanding the pathophysiology of humoral hypercalcemia of malignancy

(HHM) led to the discovery of the parathyroid hormone-related protein (PTHrP) (45). Owing to the N-terminal homology of PTH and PTHrP, both interacts with the same GPCR termed as type 1 PTH/PTHrP receptor (PTH1R). Unlike PTH which functions like a peptide hormone, PTHrP does not circulate (except during lactation and cancer) (46). In *Pthlh*$^{-/-}$ and *Pth1r*$^{-/-}$ embryos, loss of PTHrP signaling led to arrested mammary and nipple morphogenesis; while the overexpression of PTHrP (via the keratin 14 promoter) gave rise to ectopic nipples (45–47). A classic endocrine negative feedback loop is set up by CaSR signaling in the lactating breast which suppresses PTHrP production to counter bone calcium resorption, which has been proved both genetically and pharmacologically (41, 48).

CASR IN BREAST CANCER

The CaSR seems to function as an oncogene or tumor suppressor gene based on the cancer site (**Figure 1**). The expression of CaSR is greatly reduced or nullified in neuroblastomas, parathyroid

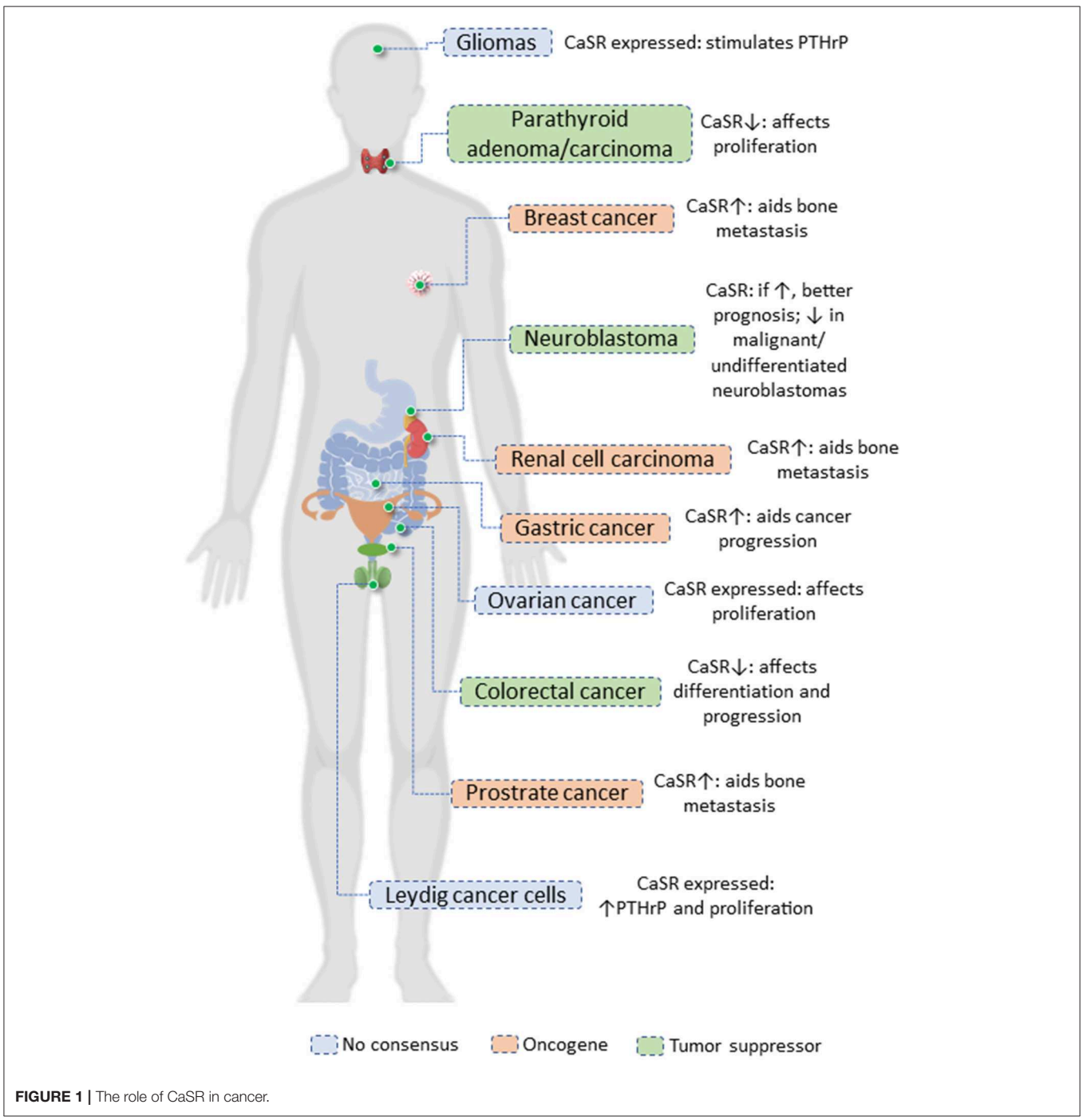

FIGURE 1 | The role of CaSR in cancer.

cancer, and colorectal cancer (49–51). In our tissue of interest, the mammary gland, most of the evidences point toward its role in promoting cancer progression. Besides this, the CaSR also acts as an oncogene in several cancers such as ovarian, prostate, and testicular cancer (52–54). Although we will be focusing on the mammary gland, it is important to keep in mind the tissues where the CaSR aids tumor progression; cumulative evidences of similar function in different tissues would help us decipher the intricacies of the signaling aspects of the CaSR.

Genetic Aspects of the CaSR

Studies have aimed to find a correlation between breast cancer risk and single nucleotide polymorphisms (SNPs) of the CaSR gene. According to a recent review, only four articles (comprising of case-control and meta-analyzes studies) point toward a significant association (55). The African-American Breast Cancer Epidemiology and Risk (AMBER) study demonstrated that the SNP in CASR rs112594756 presented with a higher odds ratio for estrogen receptor status in breast cancer (56). Li et al. found that the SNP rs17251221 was associated with reduced mRNA and protein levels of CaSR and could be a risk factor as well as a prognostic indicator of breast cancer. It is noteworthy that the same intronic polymorphism with "AG" and "GG" genotypes lowered the risk for ovarian cancer, even if it didn't correlate with survival (57, 58). CaSR SNP at rs1801725 was shown to have associated breast cancer with circulating calcium levels. Wang L. et al. showed that the decreased sensitivity of the CaSR to calcium due to the aforementioned polymorphism might predispose risk of breast cancer in up to 20% of cases with aggressive breast tumors linked to calcium in circulation (59). BRCA1 is a well-characterized tumor-suppressor gene, which is involved in various cellular functions and women who carry a mutated BRCA1 allele are at higher risk of developing breast cancer. Functional linkage studies between the CaSR and BRCA1 showed that cells harboring BRCA1 mutants with loss of expression of BRCA1 had downregulated CaSR expression. Data from these studies also showed that BRCA1 functioned through the CaSR in inhibiting survivin (an anti-apoptotic factor) expression. This means that the CaSR could rescue, in part, the deleterious effect of loss in BRCA1 function (60).

PTHrP-CaSR Axis

We already discussed the role of PTHrP in the normal functioning of the breast, but it becomes interesting how it interacts with the CaSR in contributing to pathogenesis. Some excellent research by the Wysolmerski lab led to a possible explanation of the opposing effects of CaSR on PTHrP production based on alternate G-protein coupling. Generally, PTHrP secretion is suppressed by rising calcium levels in the normal breast tissue, but it is stimulated in breast cancer cells. It was seen that the CaSR used the $G\alpha_i$ coupling in normal mammary epithelial cells but switched to $G\alpha_s$ in MCF-7 and Comma-D cells. The alternative coupling stimulated adenyl cyclase activity (as opposed to inhibition in non-transformed cells), resulting in activation of cAMP/PKA pathways which are known to regulate PTHrP gene expression and PTHrP secretion (61). Manipulating the cAMP levels independent from CaSR activity was seen to produce a similar effect in PTHrP production in the mentioned cell types (62). Activation the CaSR can also act in a concerted way with transforming growth factor β to promote PTHrP secretion, as seen in MCF-7 and MDA-MB-231 cells (48).

PTHrP was discovered to be a causal factor in HHM but has been subjected to conflicting reports in its function in primary tumors. While some reports suggest that PTHrP production by primary breast tumors is a marker of bone metastases, others, notably a large prospective study, suggested that PTHrP production by the primary tumor confers a "less invasive phenotype" and inversely correlates with bone metastases (63, 64). The PTHrP gene, on the other hand, was recently identified as a genomic locus for breast cancer susceptibility (65). However, efforts aimed at deciphering the relation between CaSR and PTHrP in breast cancer demonstrated that the action of CaSR is mediated by nuclear PTHrP and partly affects proliferation and apoptosis. When a mutant form of PTHrP which couldn't translocate into the nucleus was transduced into cells, they failed to rescue the phenotype affected by depletion of the CaSR. When either the CaSR or PTHrP was knocked down in BT474 and MDA-MB-231 cells *in vitro*, it sensitized them to cellular death in response to increasing concentrations extracellular calcium. Furthermore, ablating the CaSR in MMTV-PyMT (mouse mammary tumor virus-polyoma middle tumor-antigen) transgenic mouse model tumor cells promoted apoptosis and inhibited growth *ex vivo* (66). Mice bearing C26-DCT colon tumors treated with Cinacalcet to reduce hypercalcemia presents an interesting case as the tumor cells do not express the CaSR. The attenuation in PTHrP-mediated increase in serum Ca^{2+}, as observed in parathyroidectomized rats in which hypercalcemia had been induced with PTHrP injections, resulted from increased secretion of calcitonin and suggests calcimimetics didn't act directly on the tumor cells (67, 68).

BONE METASTASIS

Following Paget's seed and soil hypothesis, the bone microenvironment provides a fertile "soil" for breast and prostate cancer "seeding," among other carcinomas, by hosting a plethora of biochemical or physical properties that makes it attractive for tumor growth (69, 70). This metastatic niche also provides homing signals like pH and extracellular Ca^{2+}, which can be said to lure the cancer cells. Our team showed that highly bone-metastatic cells were prone to a greater migratory effect compared to less metastatic ones in response to Ca^{2+} and siRNA directed against the CaSR was able to mitigate that effect (71). Taking that hypothesis forward, we were also able to show *in vivo* that overexpression of a functional CaSR in MDA-MB-231 cells greatly increased their osteolytic potential mediated by epiregulin secretion, and downregulation of OPG (Osteoprotegerin) in osteoblastic cells, which could upregulate osteoclastogenesis (72). As adhesion also plays a key role in metastasis, Tharmalingam et al. reported for the first time that the coupling of CaSR and integrins in rat medullary thyroid carcinoma cells, along with release of $[Ca^{2+}]_i$, promotes adhesion and migration (73). This builds upon the studies which have shown that CaSR aids

metastasis and hematopoietic stem cell harboring in adult bone marrow- which are also dependent on integrin-mediated cell adhesion (74, 75).

Before cancer cells find their way into the circulation, they may have to adopt an invasive phenotype, a phenomenon referred to as the epithelial–mesenchymal transition (EMT). During this adoption, an overlooked feature of breast tumors is the biological significance of microcalcifications *in situ*, mainly comprising of calcium oxalate or hydroxyapatite (76, 77). This had been partly explored long back, where the osteotropism of breast cancer was correlated with its ability of inducing microcalcifications by expressing bone matrix proteins (78). Clinically, mammographic calcifications aid in distinguishing benign from potentially malignant changes (79). Although calcium oxalate is mostly associated with benign tumors, hydroxyapatite has been linked to both benign and malignant ones (80). Taken together, it points toward a significant role of calcium signaling. Davis et al. found that the EGF-induced EMT in MDA-MB-468 cells was calcium signal dependent (81). Activation of the CaSR in breast cancer cells have also been shown to stimulate cell proliferation acting through membrane metalloproteinases, upregulating the transient receptor potential channel 1, stimulating EGFR, and ERK1/2 phosphorylation (82, 83). The link between CaSR and EMT in breast cancer is yet to be explored, but studies have shown that inducing CaSR in colon cancer (where it acts as a tumor-suppressor) inhibits EMT and lower expression in lung adenocarcinomas promotes a mesenchymal phenotype (84–86). The estrogen receptor alpha (ER) expression in primary breast cancers is linked to incidence of bone metastases and its activity is a clinically important metric (87). It was reported that high Ca^{2+} levels which are released during tumor induced bone resorption, and specific CaSR agonists increases ER transcriptional activity and decreases ER protein levels (88). Although there is a need for confirming an unequivocal role of the CaSR in bone metastasis, we can still speculate the mechanisms by which the bone microenvironment might act through the CaSR in promoting osteolysis or bone metastases. The tumor cells needs to adapt to this Ca^{2+} rich microenvironment to proliferate and promote osteolysis, and increased PTHrP secretion might be contributing to this by its paracrine actions (61). Bone marrow stromal cells and osteoblasts express the PTH1R which binds to PTHrP produced by skeletal metastatic breast cancer cells initiating the vicious cycle and is exacerbated by the calcium-CaSR signaling. Of all the factors released during bone resorption, matrix-derived growth factors can aid tumor cell survival and/or PTHrP production (43), feeding the cycle of osteolysis and thus, investigating the CaSR-PTHrP axis might open doors on a therapeutic front. If metastatic cell growth can be halted, the tumor-bone vicious cycle can be targeted, and the bone microenvironment can be rendered inhospitable for tumor colonization- it can be the ideal therapeutic option, which makes targeting the PTHrP-CaSR axis seems so compelling.

In the "vicious cycle" fueled by tumor cells that leads to a disrupted osteoblast-osteoclast coupling, the CaSR may also serve as a target on osteoclasts. It was shown that by either antagonizing the receptor on osteoclasts or in those lacking a functional CaSR led to increased apoptosis induced by high extracellular Ca^{2+} and also impaired osteoclastogenesis. Their data also suggested that CaSR mediated NF-κB translocation to the nucleus of osteoclasts and their activation led to apoptosis of mature osteoclasts (89). Identifying the interplay between the CaSR and various factors which aid tumor cell homing, survival and proliferation in the bone microenvironment can shed light on the extent it is involved in the processes.

CALCILYTICS AND MODE OF ACTION

The definition of "calcilytics" is based on its function and doesn't account for its structure or CaSR-binding sites. The rationale for developing such compounds stemmed from the requirement of alternative small molecule calcilytics for treating osteoporosis; the standard of care at that time being anabolic therapies using PTH analogs (teriparatide) and PTH-related peptides (90). In 2001, the compound labeled as NPS-2143 became the first one to be reported in having the ability to inhibit CaSR activity (91, 92). Its potency was apparent, having an IC_{50} of 43nM in blocking Ca^{2+} accumulation in response to receptor activation (carried out in HEK 293 cells), going as high as 3 μM without affecting several other structurally similar GPCRs. Intravenous infusions in normal rats caused a rapid 4- to 5- fold increase in circulating PTH levels, and plasma Ca^{2+} levels were significantly elevated only after 90 min into the infusion with a slow return to baseline levels. Pharmacokinetic studies revealed an oral bioavailability of 11% in rats and high rate of clearance attesting to its low $t_{1/2}$ of 2 h. Additionally, a single oral dose led to a sustained increase (>4 h) in plasma PTH levels, owing to its lipophilic nature which possibly causes prolonged systemic exposure (91, 92).

Despite harboring promise, calcilytics failed in treating osteoporosis in clinical settings; it has been well-reviewed but we will summarize the findings (93). Three calcilytic compounds, namely ronacaleret, JTT-305/MK-5442, and AXT914, were able to advance into Phase II clinical trials but halted due to lack of efficacy at pre-specified interim analyses. However, they were well-tolerated and had no off-target effects that staked their safety/efficacy (92, 93). Their failure due to on-target effects on bone is because administering calcilytics is equivalent to ablating the CaSR and blocking the CaSR in bone cells has been shown to influence bone turnover (93–95). There have been many studies to show the essential role of the CaSR in osteoblast differentiation, survival, and proliferation (96). In osteoclasts, calcilytics have a different pharmacological profile, as compared to parathyroid cells, which can be due to decreasing pH in the resorption pits which makes the CaSR less responsive (96–98).

Calcilytics and Cancer

We previously stated that the CaSR acts as an oncogene in the prostrate, ovarian, and testicular cancer. In addition, Brenner lab showed that the bone metastasis caused by renal cell carcinoma (RCC) was promoted by the extracellular Ca^{2+} through the CaSR. They found that the CaSR was highly expressed in patient samples from those with bone metastases as compared to those with no or lung metastases. The calcilytic NPS-2143 acted in a predictable manner by blunting the response to calcium induced

migration and proliferation of bone metastatic primary RCC cells (99). Very recently, their lab also implicated the receptor in promoting bone metastases in mice, using 786-O (RCC) cells with stably transfected *CaSR* gene. NPS-2143 was able to inhibit the phosphorylation of SHC, AKT, ERK, JNK and p90RSK in response to high Ca^{2+} in these transfected cells (100).

An overlooked aspect of the targeting CaSR is its interplay with cytokines and growth factors, which is quite interesting given that they play a significant role in cancer. Nielsen et al. were probably the first ones who demonstrated the positive correlation between the cytokine IL-1β and CaSR mRNA levels in bovine parathyroid gland samples. They were investigating the paracrine nature of IL-1 on PTH secretion, PTH mRNA, and CaSR mRNA; IL-1β (2,000 pg/ml) upregulated CaSR mRNA levels by 180% (of control) and an IL-1 receptor antagonist abolished this effect (101). The first direct evidence of the involvement of the endogenously expressed CaSR in the secretion of multiple cytokines and growth factors by metastatic breast cancer cells was reported by Hernández-Bedolla et al. They reported that CaSR activation in MDA-MB-231 cells transactivated EGFR and stimulated the secretion of endothelial chemotactic and pro-angiogenic factors like GM-CSF (granulocyte-macrophage colony stimulating factor), EGF (epidermal growth factor), MDC (macrophage-derived chemokine), FGF-4 (fibroblast growth factor-4), and IGFBP-2 (insulin-like growth factor-binding protein-2). The receptor was also shown to diminish the constitutive secretion IL-6 and β-NGF (β-nerve growth factor). It was interesting to see that common angiogenic factors (like Vascular endothelial growth factor) and pro-inflammatory cytokines (like TNF-a, IL-1a, IL-10, and others) were not detectable in response to PAE (porcine aortic endothelial) cells in the microarrays used for screening, implying that the CaSR is selectively responsible for promoting a specific set of growth factors and cytokines. As anticipated, NPS-2143 antagonized the receptor response, inhibited the secretion of the mentioned factors, and attenuated the angiogenic effect of the breast cancer cells on PAE cells (102). An apparent paradox regarding the secretory patterns of IL-6 was addressed later by the same lab. They observed that basal activity of the endogenously expressed receptor in MDA-MB-231 cells stimulated IL-6 secretion, but agonist stimulation seemingly had an opposite effect. Agonist stimulation, with 1.85 mM Ca^{2+} and R568 (a calcimimetic), engages the CaSR in Rab11a dependent endosomal recycling and PI3K signaling pathway, crucial in inhibiting IL-6 secretion. To compare it with "normal" mammary epithelial MCF-12A cells, CaSR stimulation increased IL-6 secretion. Regardless of cell type and receptor activation, the calcilytic NPS-2143 decreased detectable IL-6 levels in the cell culture supernatants (103). The estrogen receptor (ER) activity induced by high calcium levels in ER+ breast cancer cells were also evidenced to be partly rescued with calcilytics (88). A recent report on the CaSR promoting gastric cancer progression mentions a few experiments where they used calcilytics *in vivo* to bring down tumor growth and metastasis. Mice bearing xenografted tumors injected with $CaCl_2$ or $CaCl_2$ plus NPS-2143 at the site of implantation had around 46% reduction in tumor volume with the treatment. Also, MKN45 cells pre-cocultured with or without NPS-2143 had a significantly lesser number of metastatic tumors when injected intraperitoneally in nude mice (104). This is quite intriguing as it is the first time to our knowledge where calcilytics have been used *in vivo*, albeit intratumorally, to directly target tumors where CaSR aids cancer progression. The team went on to show that there is a functional link between CaSR and human telomerase reverse transcriptase (hTERT) in gastric cancers, where calcilytics inhibited Ca-mediated upregulation of hTERT and accumulation of p-Akt (105). A more direct effect of calcilytics on gastric cancer cells was also reported, where NPS-2143 inhibited migration, invasion, proliferation, and promoted apoptosis. They also reported the upregulation of CaSR in gastric cancer cells and tissues (106). A short communication from Yamamura et al. showed that the calcilytic NPS-2143 inhibited the proliferation and migration in prostate cancer cells, suggesting their therapeutic potential (107). In all these types of cancer where the CaSR is upregulated or acts as an oncogene, like in breast cancer, the effect of calcilytics in impeding metastasis is highlighted.

Every therapeutic decision involves a risk-benefit tradeoff. The CaSR might be therapeutically challenging to target due to its ubiquity and its role in maintaining physiological functions. Systemic administration of calcilytics may result in unpredictable effects in a complex disease like breast cancer. The main risk would be exacerbating existing hypercalcemia as data amassed from the various clinical trials with calcilytics showed pre-dose serum Ca^{2+} to be elevated in many cases (93). If calcilytics are suggested as a therapy, it would be important to diagnose if the patient has hypercalcemia and whether it arose from HHM with osseous involvement, or hyperparathyroidism from adenomas (108) because calcilytics would be detrimental in the latter case. Besides this, clinical trials attest that calcilytics were devoid of any other major side-effects (90).

Calcilytics were developed to treat osteoporosis by a bone-anabolic effect and mimics an intermittent PTH treatment. Continual exposure to PTH has been shown to have catabolic effect but intermittent administration of PTH or PTHrP results in net bone formation (109–111). Swami et al. showed that this effect of intermittent PTH treatment reduced cancer cell engraftment and incidence of skeletal tumors *in vivo* in various models involving MDA-MB-231 cells or 4T1 murine human breast cancer tumors, and that it rendered a less metastatic phenotype to the cells. However, pre-treatment of mice with intermittent PTH in an orthotopic 4T1 breast cancer model didn't affect the primary tumor volume. In other pre-treatment or treatment models, the treatment reduced skeletal metastases but didn't affect metastases to other internal organs (112). This effect of intermittent PTH treatment on the bone microenvironment to hinder metastases to bones can also be a mechanism through which calcilytics could function in reducing tumor burden. Whether or not calcilytics are able to release the desired amount of PTH to reach the aforementioned effect require further investigation. The lack of tissue selectivity of calcilytics is still a challenge and it needs further development to prevent off-target effects or on-target effects in the bone, but it is interesting that it can probably infiltrate the bone microenvironment. However, when coupled to 17β-estradiol in

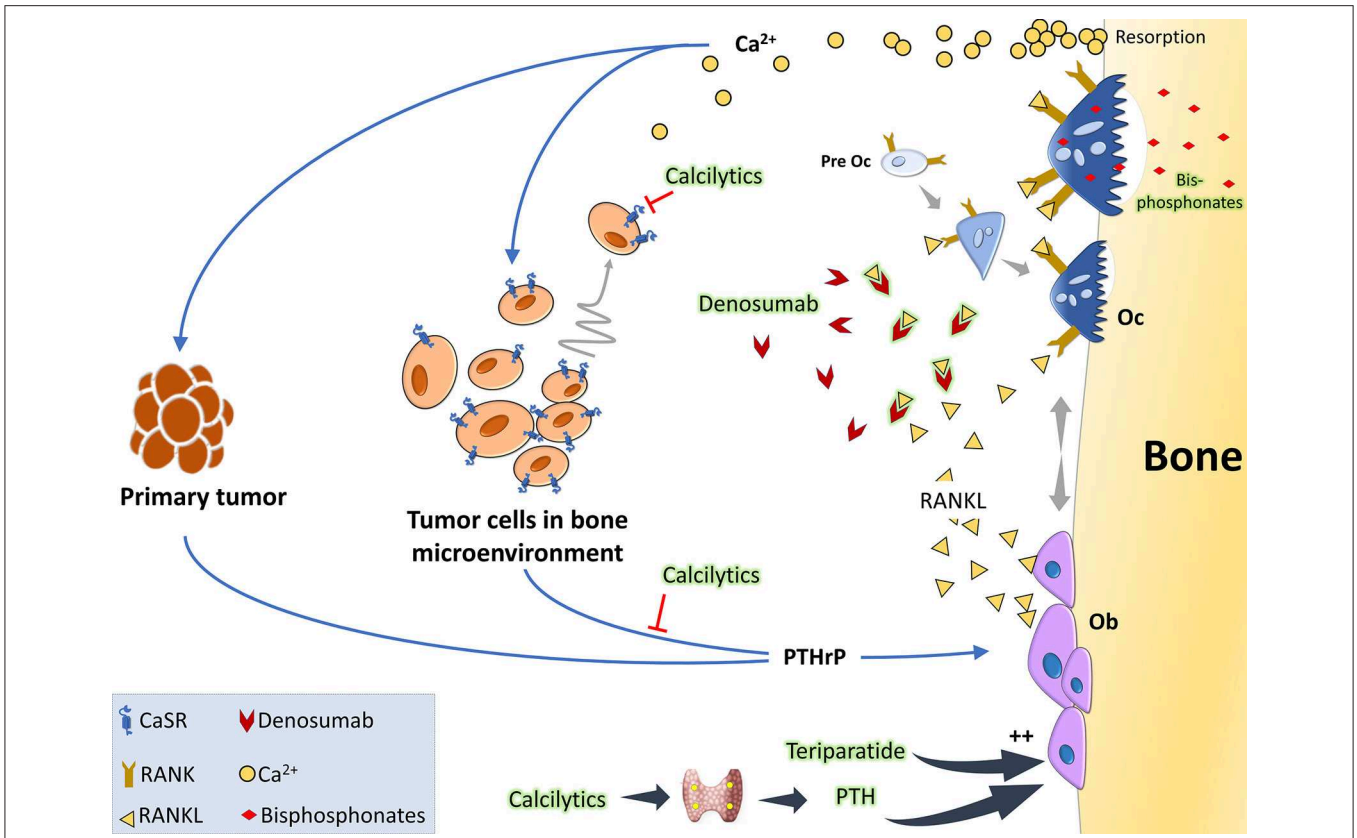

FIGURE 2 | The vicious cycle of osteolytic metastasis in breast cancer and therapeutic targets: tumor cells produce PTHrP which acts on osteoblasts (Ob) to stimulate production of RANKL. When RANKL binds to its receptor RANK, osteoclast precursor cells undergo differentiation to activated osteoclasts (Oc). These activated Oc now undergo maturation and start active resorption which releases copious amount of Ca^{2+} and growth factors. Acting via the CaSR on the tumor cells, Ca^{2+} is considered to be a chemoattractant and also further stimulates PTHrP synthesis, thereby aggravating the vicious cycle. Clinically, the standard of care is either RANKL inhibitors like denosumab which binds to it and prevents it from interacting with its receptor RANK or bisphosphonates which are metabolic poisons taken up by the osteoclasts during resorption. Anabolic therapies, like intermittent teriparatide or the intermittent PTH effect mimicked by administering calcilytics, can be a promising option as they would increase Ob activity. Calcilytics, which are CaSR antagonists, can also aid by decreasing the chemoattraction of the tumor cells to the Ca^{2+} gradient and by hindering PTHrP production.

ovariectomized rats, it led to increase in cancellous bone area (97), which opens up an interesting possibility of coupling anti-resorptives and calcilytics.

THERAPEUTIC PERSPECTIVE

The principal therapeutic strategies in the market aim at targeting osteoclasts, given that most bone metastases intersect with exacerbated osteoclast activity (**Figure 2**). Under the broad umbrella of antiresorptive therapies, one major category is a class of compounds called bisphosphonates, which in essence are metabolic poisons inducing apoptosis in osteoclasts. The prodigal drug of this class appears to be zolendronate, which has braved various clinical trials to show its effectiveness in reducing skeletal related events (SREs) and also shown to have anticancer activity when used as an adjuvant or neoadjuvant (113). Unfortunately, this therapy comes with its share of side-effects like osteonecrosis of the jaw, bone pain, hypocalcemia, and fractures. Also their high affinity for the bone makes them build up in the tissues and causes adynamic bones (114, 115). Another major category is the humanized anti-RANKL antibody, denosumab, which acts by blocking the association of RANK-RANKL and in turn blocks osteoclastogenesis. Denosumab appears to be the preferred anti-resorptive therapy in the market as phase III clinical trials showed that they are more effective in delaying SREs compared to zolendronate (116). Side-effects of this therapy include hypocalcemia, nausea, fatigue, and osteonecrosis of the jaw (116, 117). Both these therapies have also highlighted their role in antitumoral effects by aiding the antitumor immune system (118, 119). In patients with osteolytic bone diseases who are put on such therapies, the disease often progresses and in 50% of the patients there is a recurrence with SREs (120). Recent alternative therapies for targeting osteoclasts include Cathepsin-K inhibitors, c-src inhibitors, and integrin inhibitors (121–123). Some cathepsin-K inhibitors made their way to clinical trials but had to be discontinued due their side effects on skin, risk of atrial fibrillation, and stroke. There are ongoing trials with c-src inhibitors like Dasatinib and Saracatinib, and also with anti-αvβ3 integrin MABs (Etaracizumab) (113, 115).

Since metastatic cancer cells are unable to destroy the bone on their own, they hijack the bone cells to create an environment favorable for their growth leading to the dire complications associated with bone metastases. Thus, it is quite understandable why most of the drugs in the market for bone metastatic patients are bone targeted. An interesting proposition about harnessing osteoblasts, based on data from patients, *in vivo* experiments, and co cultures, suggest that osteoblasts and their secretomes were hostile to growth of myeloma cells in the bone (124, 125). Similar *in vitro* data in the case of breast cancer also showed that introduction of osteoblasts curbed bone turnover caused by osteolytic breast cancer (126). Although other therapies have been tackling cancer related bone diseases, regaining bone health and quality remains a challenge. It wouldn't be far-fetched to talk about bone anabolic agents in such cases. The most widely used anabolic agent is teriparatide (PTH 1-34) for osteoporosis and was shown to suppress myeloma growth. The use of teriparatide in the clinic on patients with cancer is highly improbable as it was shown to increase incidences of osteosarcoma in rodents- but the anti-tumor effects of PTH warrants further investigation into the use of bone anabolic agents against osteolytic breast cancer. Calcilytics can be a strong contender as there is mounting evidence toward its inhibitory effects on metastasis of cancer cells as discussed and that it has a bone anabolic effect. The risk of using such compounds are that it may exacerbate the hypercalcemia, and by blocking renal excretion of Ca^{2+}, further raise serum Ca^{2+} levels. Research on calcilytics for such indications should be aimed at increasing its tissue specificity, reducing off-target effects, targeted delivery, and finding out its interaction with current therapies in the market. A drug with direct actions on tumor cells and having a potent anabolic effect might be quite helpful in the clinic.

CONCLUSION

Since the discovery and cloning of the extracellular CaSR, we have been able to throw light on its vital role in orchestrating calcium homeostasis in the body. However, among its non-calciotropic roles, it has been shown to be expressed in various organs like the breast and implicated in numerous cellular processes like differentiation, migration, proliferation, etc. During lactation, it coordinates maternal and neonatal calcium metabolism. However, in a diseased setting, where there is rising evidence of the CaSR acting as an oncogene in breast cancer, it is said to facilitate a vicious cycle of osteolysis and tumor growth affecting the pathophysiology of bone metastases. Breast cancer is the prime cause of cancer mortality in women and although we have come a long way in diagnosis, treatment, and disease management, metastatic disease remains a major challenge. Current therapies are bone-targeted, and we present a case where CaSR antagonists can be an alternate strategy or further improve the standard-of-care. The CaSR may sit in a cascade of complex signaling events and it would be worth investigating if CaSR based agents can prevent or delay bone destruction, even though more work is required to elucidate the intricacies of its role and for producing better targeted drugs.

AUTHOR CONTRIBUTIONS

SD and RM conceived the idea for the review. SD wrote the manuscript under the guidance of RM. PC, SK, and MB contributed to substantial inputs which helped structure the review and frame the concepts.

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REFERENCES

1. Nemeth EF. Misconceptions about calcimimetics. *Ann N Y Acad Sci*. (2006) 1068:471–6. doi: 10.1196/annals.1346.044
2. McLean FC. Unsolved problems of parathyroid physiology. In: Greep RO, Talmage RV, editors. *The Parathyroids*. Springfield, IL: Charles C. Thomas (1961). p. 7–18.
3. López-Barneo J, Armstrong CM. Depolarizing response of rat parathyroid cells to divalent cations. *J Gen Physiol*. (1983) 82:269–94. doi: 10.1085/jgp.82.2.269
4. Shoback DM, Chen TH. Injection of poly(A)+ RNA from bovine parathyroid tissue into *Xenopus oocytes* confers sensitivity to extracellular calcium. *J. Bone Miner. Res*. (1991) 6:S135.
5. Racke FK, Dubyak GR, Nemeth EF. Functional expression of the parathyroid cell calcium receptor in Xenopus oocytes. *J. Bone Miner. Res*. (1991) 6:S118.
6. Brown EM, Gamba G, Riccardi D, Lombardi M, Butters R, Kifor O, et al. Cloning and characterization of an extracellular Ca^{2+}-sensing receptor from bovine parathyroid. *Nature*. (1993) 366:575–80. doi: 10.1038/366575a0
7. Pollak MR, Brown EM, Chou Y-HW, Hebert SC, Marx SJ, Stelnmann B, et al. Mutations in the human Ca^{2+}-sensing receptor gene cause familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism. *Cell*. (1993) 75:1297–303. doi: 10.1016/0092-8674(93)90617-Y
8. Zhang C, Zhang T, Zou J, Miller CL, Gorkhali R, Yang J-Y, et al. Structural basis for regulation of human calcium-sensing receptor by magnesium ions and an unexpected tryptophan derivative co-agonist. *Sci Adv*. (2016) 2:e1600241. doi: 10.1126/sciadv.1600241
9. Chakravarti B, Chattopadhyay N, Brown EM. Signaling through the extracellular Calcium-Sensing Receptor (CaSR). In: Shahidul IM, editors, *Calcium Signaling*. Dordrecht: Springer (2012). p. 103–42.
10. Nemeth EF. Regulation of cytosolic calcium by extracellular divalent cations in C-cells and parathyroid cells. *Cell Calcium*. (1990) 11:323–7. doi: 10.1016/0143-4160(90)90033-Q
11. Quinn SJ, Ye CP, Diaz R, Kifor O, Bai M, Vassilev P, et al. The Ca^{2+}-sensing receptor: a target for polyamines. *Am J Physiol*. (1997) 273:C1315–23. doi: 10.1152/ajpcell.1997.273.4.C1315
12. Brown EM, Katz C, Butters R, Kifor O. Polyarginine, polylysine, and protamine mimic the effects of high extracellular calcium concentrations

on dispersed bovine parathyroid cells. *J Bone Miner Res*. (1991) 6:1217–25. doi: 10.1002/jbmr.5650061112
13. Ye C, Ho-Pao CL, Kanazirska M, Quinn S, Rogers K, Seidman CE, et al. Amyloid-beta proteins activate Ca(2+)-permeable channels through calcium-sensing receptors. *J Neurosci Res*. (1997) 47:547–54. doi: 10.1002/(SICI)1097-4547(19970301)47:5<547::AID-JNR10>3.0.CO;2-V
14. Brown EM, Butters R, Katz C, Kifor O. Neomycin mimics the effects of high extracellular calcium concentrations on parathyroid function in dispersed bovine parathyroid cells. *Endocrinology*. (1991) 128:3047–54. doi: 10.1210/endo-128-6-3047
15. Brown E, Enyedi P, LeBoff M, Rotberg J, Preston J, Chen C. High extracellular Ca^{2+} and Mg2+ stimulate accumulation of inositol phosphates in bovine parathyroid cells. *FEBS Lett*. (1987) 218:113–8. doi: 10.1016/0014-5793(87)81029-3
16. Wettschureck N, Lee E, Libutti SK, Offermanns S, Robey PG, Spiegel AM. Parathyroid-specific double knockout of Gq and G11 alpha-subunits leads to a phenotype resembling germline knockout of the extracellular Ca^{2+} -sensing receptor. *Mol Endocrinol*. (2007) 21:274–80. doi: 10.1210/me.2006-0110
17. Hannan FM, Babinsky VN, Thakker RV. Disorders of the calcium-sensing receptor and partner proteins: insights into the molecular basis of calcium homeostasis. *J Mol Endocrinol*. (2016) 57:R127–42. doi: 10.1530/JME-16-0124
18. Brennan T, Rybchyn M, Green W, Atwa S, Conigrave A, Mason R. Osteoblasts play key roles in the mechanisms of action of strontium ranelate. *Br J Pharmacol*. (2009) 157:1291–300. doi: 10.1111/j.1476-5381.2009.00305.x
19. Rybchyn MS, Slater M, Conigrave AD, Mason RS. An Akt-dependent increase in canonical Wnt signaling and a decrease in sclerostin protein levels are involved in strontium ranelate-induced osteogenic effects in human osteoblasts. *J Biol Chem*. (2011) 286:23771–9. doi: 10.1074/jbc.M111.251116
20. Worzfeld T, Wettschureck N, Offermanns S. G12/G13-mediated signalling in mammalian physiology and disease. *Trends Pharmacol Sci*. (2008) 29:582–9. doi: 10.1016/j.tips.2008.08.002
21. Ward DT, Riccardi D. New concepts in calcium-sensing receptor pharmacology and signalling. *Br J Pharmacol*. (2012) 165:35–48. doi: 10.1111/j.1476-5381.2011.01511.x
22. Brennan SC, Thiem U, Roth S, Aggarwal A, Fetahu ISh, Tennakoon S, et al. Calcium sensing receptor signalling in physiology and cancer. *Biochim Biophys Acta*. (2013) 1833:1732–44. doi: 10.1016/j.bbamcr.2012.12.011
23. Mamillapalli R, Wysolmerski J. The calcium-sensing receptor couples to Gαs and regulates PTHrP and ACTH secretion in pituitary cells. *J Endocrinol*. (2010) 204:287–97. doi: 10.1677/JOE-09-0183
24. Brennan SC, Wilkinson WJ, Tseng H-E, Finney B, Monk B, Dibble H, et al. The extracellular calcium-sensing receptor regulates human fetal lung development via CFTR. *Sci Rep*. (2016) 6. doi: 10.1038/srep21975
25. Conigrave AD, Ward DT. Calcium-sensing receptor (CaSR): pharmacological properties and signaling pathways. *Best Pract Res Clin Endocrinol Metab*. (2013) 27:315–31. doi: 10.1016/j.beem.2013.05.010
26. Thomsen ARB, Smajilovic S, Bräuner-Osborne H. Novel strategies in drug discovery of the calcium-sensing receptor based on biased signaling. *Curr Drug Targets*. (2012) 13:1324–35. doi: 10.2174/138945012802429642
27. Leach K, Conigrave AD, Sexton PM, Christopoulos A. Towards tissue-specific pharmacology: insights from the calcium-sensing receptor as a paradigm for GPCR (patho)physiological bias. *Trends Pharmacol Sci*. (2015) 36:215–25. doi: 10.1016/j.tips.2015.02.004
28. Hofer AM, Brown EM. Calcium: extracellular calcium sensing and signalling. *Nat Rev Mol Cell Biol*. (2003) 4:530–8. doi: 10.1038/nrm1154
29. Pollak MR, Brown EM, Estep HL, McLaine PN, Kifor O, Park J, et al. Autosomal dominant hypocalcaemia caused by a Ca^{2+}-sensing receptor gene mutation. *Nat Genet*. (1994) 8:303–7. doi: 10.1038/ng1194-303
30. Watanabe S, Fukumoto S, Chang H, Takeuchi Y, Hasegawa Y, Okazaki R, et al. Association between activating mutations of calcium-sensing receptor and Bartter's syndrome. *Lancet*. (2002) 360:692–4. doi: 10.1016/S0140-6736(02)09842-2
31. Alfadda TI, Saleh AMA, Houillier P, Geibel JP. Calcium-sensing receptor 20 years later. *Am J Physiol Cell Physiol*. (2014) 307:C221–31. doi: 10.1152/ajpcell.00139.2014
32. Aida K, Koishi S, Tawata M, Onaya T. Molecular cloning of a putative Ca(2+)-sensing receptor cDNA from human kidney. *Biochem Biophys Res Commun*. (1995) 214:524–9. doi: 10.1006/bbrc.1995.2318
33. Ray JM, Squires PE, Curtis SB, Meloche MR, Buchan AM. Expression of the calcium-sensing receptor on human antral gastrin cells in culture. *J Clin Invest*. (1997) 99:2328–33. doi: 10.1172/JCI119413
34. Chattopadhyay N, Cheng I, Rogers K, Riccardi D, Hall A, Diaz R, et al. Identification and localization of extracellular Ca(2+)-sensing receptor in rat intestine. *Am J Physiol*. (1998) 274:G122–30. doi: 10.1152/ajpgi.1998.274.1.G122
35. Bikle DD, Ratnam A, Mauro T, Harris J, Pillai S. Changes in calcium responsiveness and handling during keratinocyte differentiation. Potential role of the calcium receptor. *J Clin Invest*. (1996) 97:1085–93. doi: 10.1172/JCI118501
36. Tu C-L, Chang W, Xie Z, Bikle DD. Inactivation of the calcium sensing receptor inhibits E-cadherin-mediated cell-cell adhesion and calcium-induced differentiation in human epidermal keratinocytes. *J Biol Chem*. (2008) 283:3519–28. doi: 10.1074/jbc.M708318200
37. Ruat M, Molliver ME, Snowman AM, Snyder SH. Calcium sensing receptor: molecular cloning in rat and localization to nerve terminals. *Proc Natl Acad Sci USA*. (1995) 92:3161–5. doi: 10.1073/pnas.92.8.3161
38. Canaff L, Petit JL, Kisiel M, Watson PH, Gascon-Barré M, Hendy GN. Extracellular calcium-sensing receptor is expressed in rat hepatocytes. coupling to intracellular calcium mobilization and stimulation of bile flow. *J Biol Chem*. (2001) 276:4070–9. doi: 10.1074/jbc.M009317200
39. Tfelt-Hansen J, Hansen JL, Smajilovic S, Terwilliger EF, Haunso S, Sheikh SP. Calcium receptor is functionally expressed in rat neonatal ventricular cardiomyocytes. *Am J Physiol Heart Circ Physiol*. (2006) 290:H1165–71. doi: 10.1152/ajpheart.00821.2005
40. Cheng I, Klingensmith ME, Chattopadhyay N, Kifor O, Butters RR, Soybel DI, et al. Identification and localization of the extracellular calcium-sensing receptor in human breast. *J Clin Endocrinol Metab*. (1998) 83:703–7. doi: 10.1210/jcem.83.2.4558
41. VanHouten J, Dann P, McGeoch G, Brown EM, Krapcho K, Neville M, et al. The calcium-sensing receptor regulates mammary gland parathyroid hormone–related protein production and calcium transport. *J Clin Invest*. (2004) 113:598–608. doi: 10.1172/JCI200418776
42. VanHouten JN, Neville MC, Wysolmerski JJ. The calcium-sensing receptor regulates plasma membrane calcium adenosine triphosphatase isoform 2 activity in mammary epithelial cells: a mechanism for calcium-regulated calcium transport into milk. *Endocrinology*. (2007) 148:5943–54. doi: 10.1210/en.2007-0850
43. Kim W, Wysolmerski JJ. Calcium-sensing receptor in breast physiology and cancer. *Front Physiol*. (2016) 7:440. doi: 10.3389/fphys.2016.00440
44. Mamillapalli R, VanHouten J, Dann P, Bikle D, Chang W, Brown E, et al. Mammary-specific ablation of the calcium-sensing receptor during lactation alters maternal calcium metabolism, milk calcium transport, and neonatal calcium accrual. *Endocrinology*. (2013) 154:3031–42. doi: 10.1210/en.2012-2195
45. Broadus AE, Mangin M, Ikeda K, Insogna KL, Weir EC, Burtis WJ, et al. Humoral hypercalcemia of cancer. Identification of a novel parathyroid hormone-like peptide. *N Engl J Med*. (1988) 319:556–63. doi: 10.1056/NEJM198809013190906
46. Hiremath M, Wysolmerski J. Role of PTHrP in mammary gland development and breast cancer. *Clin Rev Bone Miner Metab*. (2014) 12:178–89. doi: 10.1007/s12018-014-9170-9
47. Jüppner H, Abou-Samra AB, Freeman M, Kong XF, Schipani E, Richards J, et al. A G protein-linked receptor for parathyroid hormone and parathyroid hormone-related peptide. *Science*. (1991) 254:1024–6. doi: 10.1126/science.1658941
48. Sanders JL, Chattopadhyay N, Kifor O, Yamaguchi T, Butters RR, Brown EM. Extracellular calcium-sensing receptor expression and its potential role in regulating parathyroid hormone-related peptide secretion in human breast cancer cell lines. *Endocrinology*. (2000) 141:4357–64. doi: 10.1210/endo.141.12.7849

49. Casalà C, Gil-Guiñón E, Ordóñez JL, Miguel-Queralt S, Rodríguez E, Galván P, et al. The calcium-sensing receptor is silenced by genetic and epigenetic mechanisms in unfavorable neuroblastomas and its reactivation induces ERK1/2-dependent apoptosis. *Carcinogenesis*. (2013) 34:268–76. doi: 10.1093/carcin/bgs338
50. Haven CJ, van Puijenbroek M, Karperien M, Fleuren G-J, Morreau H. Differential expression of the calcium sensing receptor and combined loss of chromosomes 1q and 11q in parathyroid carcinoma. *J Pathol*. (2004) 202:86–94. doi: 10.1002/path.1489
51. Fetahu IS, Höbaus J, Aggarwal A, Hummel DM, Tennakoon S, Mesteri I, et al. Calcium-sensing receptor silencing in colorectal cancer is associated with promoter hypermethylation and loss of acetylation on histone 3. *Int J Cancer*. (2014) 135:2014–23. doi: 10.1002/ijc.28856
52. Hobson SA, Wright J, Lee F, McNeil SE, Bilderback T, Rodland KD. Activation of the MAP kinase cascade by exogenous calcium-sensing receptor. *Mol Cell Endocrinol*. (2003) 200:189–98. doi: 10.1016/S0303-7207(01)00749-3
53. Feng J, Xu X, Li B, Brown E, Farris AB, Sun S-Y, et al. Prostate cancer metastatic to bone has higher expression of the calcium-sensing receptor (CaSR) than primary prostate cancer. *Receptors Clin Investig*. (2014) 1:e270. doi: 10.14800/rci.270
54. Tfelt-Hansen J, Schwarz P, Terwilliger EF, Brown EM, Chattopadhyay N. Calcium-sensing receptor induces messenger ribonucleic acid of human securin, pituitary tumor transforming gene, in rat testicular cancer. *Endocrinology*. (2003) 144:5188–93. doi: 10.1210/en.2003-0520
55. Campos-Verdes LM, Costa-Silva DR, Silva-Sampaio JP da, Barros-Oliveira M da C, Escórcio-Dourado CS, Martins LM, et al. Review of polymorphism of the calcium-sensing receptor gene and breast cancer risk. *Cancer Invest*. (2018) 36:1–7. doi: 10.1080/07357907.2018.1430817
56. Yao S, Haddad SA, Hu Q, Liu S, Lunetta KL, Ruiz-Narvaez EA, et al. Genetic variations in vitamin D-related pathways and breast cancer risk in African American women in the AMBER consortium. *Int J Cancer*. (2016) 138:2118–26. doi: 10.1002/ijc.29954
57. Li X, Kong X, Jiang L, Ma T, Yan S, Yuan C, et al. A genetic polymorphism (rs17251221) in the calcium-sensing receptor is associated with breast cancer susceptibility and prognosis. *Cell Physiol Biochem*. (2014) 33:165–72. doi: 10.1159/000356659
58. Yan S, Yuan C, Yang Q, Li X, Yang N, Liu X, et al. A genetic polymorphism (rs17251221) in the calcium-sensing receptor is associated with ovarian cancer susceptibility. *Oncol Rep*. (2015) 34:2151–5. doi: 10.3892/or.2015.4179
59. Wang L, Widatalla SE, Whalen DS, Ochieng J, Sakwe AM. Association of calcium sensing receptor polymorphisms at rs1801725 with circulating calcium in breast cancer patients. *BMC Cancer*. (2017) 17:511. doi: 10.1186/s12885-017-3502-3
60. Promkan M, Liu G, Patmasiriwat P, Chakrabarty S. BRCA1 suppresses the expression of survivin and promotes sensitivity to paclitaxel through the calcium sensing receptor (CaSR) in human breast cancer cells. *Cell Calcium*. (2011) 49:79–88. doi: 10.1016/j.ceca.2011.01.003
61. Mamillapalli R, VanHouten J, Zawalich W, Wysolmerski J. Switching of G-protein usage by the calcium-sensing receptor reverses its effect on parathyroid hormone-related protein secretion in normal versus malignant breast cells. *J Biol Chem*. (2008) 283:24435–47. doi: 10.1074/jbc.M801738200
62. Chilco PJ, Leopold V, Zajac JD. Differential regulation of the parathyroid hormone-related protein gene P1 and P3 promoters by cAMP. *Mol Cell Endocrinol*. (1998) 138:173–84. doi: 10.1016/S0303-7207(97)00239-6
63. Takagaki K, Takashima T, Onoda N, Tezuka K, Noda E, Kawajiri H, et al. Parathyroid hormone-related protein expression, in combination with nodal status, predicts bone metastasis and prognosis of breast cancer patients. *Exp Ther Med*. (2012) 3:963–8. doi: 10.3892/etm.2012.521
64. Henderson MA, Danks JA, Slavin JL, Byrnes GB, Choong PFM, Spillane JB, et al. Parathyroid hormone-related protein localization in breast cancers predict improved prognosis. *Cancer Res*. (2006) 66:2250–6. doi: 10.1158/0008-5472.CAN-05-2814
65. Ghoussaini M, Fletcher O, Michailidou K, Turnbull C, Schmidt MK, Dicks E, et al. Genome-wide association analysis identifies three new breast cancer susceptibility loci. *Nat Genet*. (2012) 44:312–8. doi: 10.1038/ng.1049
66. Kim W, Takyar FM, Swan K, Jeong J, VanHouten J, Sullivan C, et al. Calcium-sensing receptor promotes breast cancer by stimulating intracrine actions of parathyroid hormone–related protein. *Cancer Res*. (2016) 76:5348–60. doi: 10.1158/0008-5472.CAN-15-2614
67. Yang Y, Wang B. PTH1R-CaSR cross talk: new treatment options for breast cancer osteolytic bone metastases. *Int J Endocrinol*. (2018) 2018:8. doi: 10.1155/2018/7120979
68. Colloton M, Shatzen E, Wiemann B, Starnes C, Scully S, Henley C, et al. Cinacalcet attenuates hypercalcemia observed in mice bearing either Rice H-500 Leydig cell or C26-DCT colon tumors. *Eur J Pharmacol*. (2013) 712:8–15. doi: 10.1016/j.ejphar.2013.04.013
69. Paget S. The distribution of secondary growths in cancer of the breast. 1889. *Cancer Metastasis Rev*. (1989) 8:98–101.
70. Guise T. Examining the metastatic niche: targeting the microenvironment. *Semin Oncol*. (2010) 37(Suppl. 2):S2–14. doi: 10.1053/j.seminoncol.2010.10.007
71. Saidak Z, Boudot C, Abdoune R, Petit L, Brazier M, Mentaverri R, et al. Extracellular calcium promotes the migration of breast cancer cells through the activation of the calcium sensing receptor. *Exp Cell Res*. (2009) 315:2072–80. doi: 10.1016/j.yexcr.2009.03.003
72. Boudot C, Hénaut L, Thiem U, Geraci S, Galante M, Saldanha P, et al. Overexpression of a functional calcium-sensing receptor dramatically increases osteolytic potential of MDA-MB-231 cells in a mouse model of bone metastasis through epiregulin-mediated osteoprotegerin downregulation. *Oncotarget*. (2017) 8:56460. doi: 10.18632/oncotarget.16999
73. Tharmalingam S, Daulat AM, Antflick JE, Ahmed SM, Nemeth EF, Angers S, et al. Calcium-sensing receptor modulates cell adhesion and migration via integrins. *J Biol Chem*. (2011) 286:40922–33. doi: 10.1074/jbc.M111.265454
74. Saidak Z, Mentaverri R, Brown EM. The role of the calcium-sensing receptor in the development and progression of cancer. *Endocr Rev*. (2009) 30:178–95. doi: 10.1210/er.2008-0041
75. Lam BS, Cunningham C, Adams GB. Pharmacologic modulation of the calcium-sensing receptor enhances hematopoietic stem cell lodgment in the adult bone marrow. *Blood*. (2011) 117:1167–75. doi: 10.1182/blood-2010-05-286294
76. Morgan MP, Cooke MM, McCarthy GM. Microcalcifications associated with breast cancer: an epiphenomenon or biologically significant feature of selected tumors? *J Mammary Gland Biol Neoplasia*. (2005) 10:181–7. doi: 10.1007/s10911-005-5400-6
77. Rominger MB, Steinmetz C, Westerman R, Ramaswamy A, Albert U-S. microcalcification-associated breast cancer: presentation, successful first excision, long-term recurrence and survival rate. *Breast Care*. (2015) 10:380–5. doi: 10.1159/000440794
78. Bellahcène A, Castronovo V. Expression of bone matrix proteins in human breast cancer: potential roles in microcalcification formation and in the genesis of bone metastases. *Bull Cancer*. (1997) 84:17–24.
79. Wilkinson L, Thomas V, Sharma N. Microcalcification on mammography: approaches to interpretation and biopsy. *Br J Radiol*. (2019) 90:2016094. doi: 10.1259/bjr.20160594
80. Cox RF, Jenkinson A, Pohl K, O'Brien FJ, Morgan MP. Osteomimicry of mammary adenocarcinoma cells *in vitro*; increased expression of bone matrix proteins and proliferation within a 3D collagen environment. *PLoS ONE*. (2012) 7:e41679. doi: 10.1371/journal.pone.0041679
81. Davis FM, Azimi I, Faville RA, Peters AA, Jalink K, Putney JW, et al. Induction of epithelial-mesenchymal transition (EMT) in breast cancer cells is calcium signal dependent. *Oncogene*. (2014) 33:2307–16. doi: 10.1038/onc.2013.187
82. El Hiani Y, Ahidouch A, Lehen'kyi V, Hague F, Gouilleux F, Mentaverri R, et al. Extracellular signal-regulated kinases 1 and 2 and TRPC1 channels are required for calcium-sensing receptor-stimulated MCF-7 breast cancer cell proliferation. *Cell Physiol Biochem*. (2009) 23:335–46. doi: 10.1159/000218179
83. El Hiani Y, Lehen'kyi V, Ouadid-Ahidouch H, Ahidouch A. Activation of the calcium-sensing receptor by high calcium induced breast cancer cell proliferation and TRPC1 cation channel over-expression potentially through EGFR pathways. *Arch Biochem Biophys*. (2009) 486:58–63. doi: 10.1016/j.abb.2009.03.010
84. Aggarwal A, Prinz-Wohlgenannt M, Gröschel C, Tennakoon S, Meshcheryakova A, Chang W, et al. The calcium-sensing receptor suppresses epithelial-to-mesenchymal transition and stem cell- like phenotype in

the colon. *Molecular Cancer*. (2015) 14:61. doi: 10.1186/s12943-015-0330-4
85. Singh N, Aslam MN, Varani J, Chakrabarty S. Induction of calcium sensing receptor in human colon cancer cells by calcium, vitamin D and aquamin: promotion of a more differentiated, less malignant and indolent phenotype. *Mol Carcinogen*. (2015) 54:543–53. doi: 10.1002/mc.22123
86. Wen L, Sun L, Xi Y, Chen X, Xing Y, Sun W, et al. Expression of calcium sensing receptor and E-cadherin correlated with survival of lung adenocarcinoma. *Thoracic Cancer*. (2015) 6:754–60. doi: 10.1111/1759-7714.12255
87. Hua H, Zhang H, Kong Q, Jiang Y. Mechanisms for estrogen receptor expression in human cancer. *Exp Hematol Oncol*. (2018) 7:24. doi: 10.1186/s40164-018-0116-7
88. Journé F, Dumon J-C, Kheddoumi N, Fox J, Laios I, Leclercq G, et al. Extracellular calcium downregulates estrogen receptor alpha and increases its transcriptional activity through calcium-sensing receptor in breast cancer cells. *Bone*. (2004) 35:479–88. doi: 10.1016/j.bone.2004.03.021
89. Mentaverri R, Yano S, Chattopadhyay N, Petit L, Kifor O, Kamel S, et al. The calcium sensing receptor is directly involved in both osteoclast differentiation and apoptosis. *FASEB J*. (2006) 20:2562–4. doi: 10.1096/fj.06-6304fje
90. Nemeth EF. Anabolic therapy for osteoporosis: calcilytics. *IBMS BoneKEy*. (2008) 5:196–208. doi: 10.1138/20080318
91. Nemeth EF, Delmar EG, Heaton WL, Miller MA, Lambert LD, Conklin RL, et al. Calcilytic compounds: potent and selective Ca^{2+} receptor antagonists that stimulate secretion of parathyroid hormone. *J Pharmacol Exp Ther*. (2001) 299:323−31.
92. Nemeth EF, Van Wagenen BC, Balandrin MF. Chapter one - discovery and development of calcimimetic and calcilytic compounds. In: Witty DR, Cox B, editors. *Progress in Medicinal Chemistry*. Elsevier (2018). p. 1–86. doi: 10.1016/bs.pmch.2017.12.001
93. Nemeth EF, Goodman WG. Calcimimetic and calcilytic drugs: feats, flops, and futures. *Calcif Tissue Int*. (2016) 98:341–58. doi: 10.1007/s00223-015-0052-z
94. Dvorak MM, Siddiqua A, Ward DT, Carter DH, Dallas SL, Nemeth EF, et al. Physiological changes in extracellular calcium concentration directly control osteoblast function in the absence of calciotropic hormones. *Proc Natl Acad Sci USA*. (2004) 101:5140–5. doi: 10.1073/pnas.0306141101
95. Goltzman D, Hendy GN. The calcium-sensing receptor in bone—mechanistic and therapeutic insights. *Nat Rev Endocrinol*. (2015) 11:298–307. doi: 10.1038/nrendo.2015.30
96. Santa Maria C, Cheng Z, Li A, Wang J, Shoback D, Tu C-L, et al. Interplay between CaSR and PTH1R signaling in skeletal development and osteoanabolism. *Semin Cell Dev Biol*. (2016) 49:11–23. doi: 10.1016/j.semcdb.2015.12.004
97. Gowen M, Stroup GB, Dodds RA, James IE, Votta BJ, Smith BR, et al. Antagonizing the parathyroid calcium receptor stimulates parathyroid hormone secretion and bone formation in osteopenic rats. *J Clin Invest*. (2000) 105:1595–604. doi: 10.1172/JCI9038
98. Quinn SJ, Bai M, Brown EM. pH sensing by the calcium-sensing receptor. *J Biol Chem*. (2004) 279:37241–9. doi: 10.1074/jbc.M404520200
99. Joeckel E, Haber T, Prawitt D, Junker K, Hampel C, Thüroff JW, et al. High calcium concentration in bones promotes bone metastasis in renal cell carcinomas expressing calcium-sensing receptor. *Molecular Cancer*. (2014) 13:42. doi: 10.1186/1476-4598-13-42
100. Frees S, Breuksch I, Haber T, Bauer H-K, Chavez-Munoz C, Raven P, et al. Calcium-sensing receptor (CaSR) promotes development of bone metastasis in renal cell carcinoma. *Oncotarget*. (2018) 9:15766–79. doi: 10.18632/oncotarget.24607
101. Nielsen PK, Rasmussen AK, Butters R, Feldt-Rasmussen U, Bendtzen K, Diaz R, et al. Inhibition of PTH secretion by interleukin-1 beta in bovine parathyroid glands *in vitro* is associated with an up-regulation of the calcium-sensing receptor mRNA. *Biochem Biophys Res Commun*. (1997) 238:880–5. doi: 10.1006/bbrc.1997.7207
102. Hernández-Bedolla MA, Carretero-Ortega J, Valadez-Sánchez M, Vázquez-Prado J, Reyes-Cruz G. Chemotactic and proangiogenic role of calcium sensing receptor is linked to secretion of multiple cytokines and growth factors in breast cancer MDA-MB-231 cells. *Biochim Biophys Acta*. (2015) 1853:166–82. doi: 10.1016/j.bbamcr.2014.10.011
103. Hernández-Bedolla MA, González-Domínguez E, Zavala-Barrera C, Gutiérrez-López TY, Hidalgo-Moyle JJ, Vázquez-Prado J, et al. Calcium-sensing-receptor (CaSR) controls IL-6 secretion in metastatic breast cancer MDA-MB-231 cells by a dual mechanism revealed by agonist and inverse-agonist modulators. *Mol Cell Endocrinol*. (2016) 436:159–68. doi: 10.1016/j.mce.2016.07.038
104. Xie R, Xu J, Xiao Y, Wu J, Wan H, Tang B, et al. Calcium promotes human gastric cancer via a novel coupling of calcium-sensing receptor and TRPV4 channel. *Cancer Res*. (2017) 77:6499–512. doi: 10.1158/0008-5472.CAN-17-0360
105. Xie R, Tuo B, Yang S, Chen X-Q, Xu J. Calcium-sensing receptor bridges calcium and telomerase reverse transcriptase in gastric cancers via Akt. *Clin Transl Oncol*. (2019). doi: 10.1007/s12094-019-02226-4
106. Zhang Z-L, Li Z-R, Li J-S, Wang S-R. Calcium-sensing receptor antagonist NPS-2143 suppresses proliferation and invasion of gastric cancer cells. *Cancer Gene Ther*. (2019). doi: 10.1038/s41417-019-0128-4
107. Yamamura A, Nayeem MJ, Sato M. Calcilytics inhibit the proliferation and migration of human prostate cancer PC-3 cells. *J Pharmacol Sci*. (2019) 139:254–7. doi: 10.1016/j.jphs.2019.01.008
108. Sigvaldason H, Obayan A, von Kuster K, Pathak KA. Hypercalcemia in metastatic breast cancer unrelated to skeletal metastasis. *CMAJ*. (2016) 188:E91–4. doi: 10.1503/cmaj.150638
109. Horwitz MJ, Tedesco MB, Sereika SM, Prebehala L, Gundberg CM, Hollis BW, et al. A 7-day continuous infusion of PTH or PTHrP suppresses bone formation and uncouples bone turnover. *J Bone Miner Res*. (2011) 26:2287–97. doi: 10.1002/jbmr.415
110. Neer RM, Arnaud CD, Zanchetta JR, Prince R, Gaich GA, Reginster JY, et al. Effect of parathyroid hormone (1–34) on fractures and bone mineral density in postmenopausal women with osteoporosis. *N Engl J Med*. (2001) 344:1434–41. doi: 10.3171/foc.2001.11.2.8
111. Miller PD, Hattersley G, Riis BJ, Williams GC, Lau E, Russo LA, et al. Effect of abaloparatide vs. placebo on new vertebral fractures in postmenopausal women with osteoporosis: a randomized clinical trial. *JAMA*. (2016) 316:722–33. doi: 10.1001/jama.2016.11136
112. Swami S, Johnson J, Bettinson LA, Kimura T, Zhu H, Albertelli MA, et al. Prevention of breast cancer skeletal metastases with parathyroid hormone. *JCI Insight*. (2017) 2:90874. doi: 10.1172/jci.insight.90874
113. Sousa S, Clézardin P. Bone-targeted therapies in cancer-induced bone disease. *Calcif Tissue Int*. (2018) 102:227–50. doi: 10.1007/s00223-017-0353-5
114. Reyes C, Hitz M, Prieto-Alhambra D, Abrahamsen B. Risks and benefits of bisphosphonate therapies. *J Cell Biochem*. (2016) 117:20–8. doi: 10.1002/jcb.25266
115. Maurizi A, Rucci N. The osteoclast in bone metastasis: player and target. *Cancers*. (2018) 10:218. doi: 10.3390/cancers10070218
116. Rosen LS, Gordon D, Tchekmedyian S, Yanagihara R, Hirsh V, Krzakowski M, et al. Zoledronic acid versus placebo in the treatment of skeletal metastases in patients with lung cancer and other solid tumors: a phase III, double-blind, randomized trial–the Zoledronic Acid Lung Cancer and Other Solid Tumors Study Group. *J Clin Oncol*. (2003) 21:3150–7. doi: 10.1200/JCO.2003.04.105
117. Body J-J, Bone HG, de Boer RH, Stopeck A, Van Poznak C, Damião R, et al. Hypocalcaemia in patients with metastatic bone disease treated with denosumab. *Eur J Cancer*. (2015) 51:1812–21. doi: 10.1016/j.ejca.2015.05.016
118. Lüftner D, Niepel D, Steger GG. Therapeutic approaches for protecting bone health in patients with breast cancer. *Breast*. (2018) 37:28–35. doi: 10.1016/j.breast.2017.10.007
119. Zekri J, Mansour M, Karim SM. The anti-tumour effects of zoledronic acid. *J Bone Oncol*. (2014) 3:25–35. doi: 10.1016/j.jbo.2013.12.001
120. Coleman RE, Major P, Lipton A, Brown JE, Lee K-A, Smith M, et al. Predictive value of bone resorption and formation markers in cancer patients with bone metastases receiving the bisphosphonate zoledronic acid. *J Clin Oncol*. (2016) 23:4925–35. doi: 10.1200/JCO.2005.06.091
121. Jensen AB, Wynne C, Ramirez G, He W, Song Y, Berd Y, et al. The cathepsin K inhibitor odanacatib suppresses bone resorption in women with

breast cancer and established bone metastases: results of a 4-week, double-blind, randomized, controlled trial. *Clin Breast Cancer*. (2010) 10:452–8. doi: 10.3816/CBC.2010.n.059

122. Yang JC, Bai L, Yap S, Gao AC, Kung H-J, Evans CP. Effect of the specific Src family kinase inhibitor saracatinib on osteolytic lesions using the PC-3 bone model. *Mol Cancer Ther*. (2010) 9:1629–37. doi: 10.1158/1535-7163.MCT-09-1058
123. Carter RZ, Micocci KC, Natoli A, Redvers RP, Paquet-Fifield S, Martin ACBM, et al. Tumour but not stromal expression of β3 integrin is essential, and is required early, for spontaneous dissemination of bone-metastatic breast cancer. *J Pathol*. (2015) 235:760–72. doi: 10.1002/path.4490
124. Yaccoby S, Wezeman MJ, Zangari M, Walker R, Cottler-Fox M, Gaddy D, et al. Inhibitory effects of osteoblasts and increased bone formation on myeloma in novel culture systems and a myelomatous mouse model. *Haematologica*. (2006) 91:192–9.
125. Adamik J, Galson DL, Roodman GD. Osteoblast suppression in multiple myeloma bone disease. *J Bone Oncol*. (2018) 13:62–70. doi: 10.1016/j.jbo.2018.09.001
126. Krawetz R, Wu YE, Rancourt DE, Matyas J. Osteoblasts suppress high bone turnover caused by osteolytic breast cancer *in vitro*. *Exp Cell Res*. (2009) 315:2333–42. doi: 10.1016/j.yexcr.2009.04.026

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Role of mTOR and VEGFR Inhibition in Prevention of Metastatic Tumor Growth in the Spine

Tobias Kratzsch[1], *Andras Piffko*[1], *Thomas Broggini*[2], *Marcus Czabanka*[1] *and Peter Vajkoczy*[1]*

[1] Department of Neurosurgery, Charité University Hospital, Berlin, Germany, [2] Department of Physics, University of California, San Diego, La Jolla, CA, United States

Objective: Spinal metastatic disease remains a major problem of oncological diseases. Patients affected may suffer pain, spinal instability, and severe neurological deficits. Today, palliative surgery and radiotherapy are the mainstays of therapy. In contrast, preventive treatment strategies or treatment concepts for an early stage are lacking. Here, we have used a syngeneic, experimental spine metastases model in the mouse to test the efficacy of mTOR inhibition and anti-angiogenesis on the formation and progression of spinal melanoma metastases.

Methods: We used our previously established syngeneic spinal metastases mouse model by injecting luciferin-transfected B16 melanoma cells into the common carotid artery. Following injection, mice were treated with everolimus, an inhibitor of the mammalian target of rapamycin (mTOR) complex, axitinib, a tyrosine kinase inhibitor, that blocks vascular endothelial growth factor receptors (VEGFR) 1-3, as well as placebo. Animals were followed-up daily by neurological assessment and by repeat *in vivo* bioluminescence imaging. With occurrence of neurological deficits, a spinal MRI was performed, and mice were sacrificed. The whole spine was dissected free and analyzed by immunohistochemical techniques.

Results: Overall survival was 23 days in the control group, significantly prolonged to 30 days ($p = 0.04$) in the everolimus group, and to 28 days ($p = 0.04$) in the axitinib group. While 78% of mice in the placebo group developed symptomatic metastatic epidural spinal cord compression, only 50% did so in the treatment groups. The mean time to manifestation of paralysis was 22 days in the control group, 26 days ($p = 0.10$) in the everolimus group, and 27 days ($p = 0.06$) in the axitinib group. Screening for spinal metastases by bioluminescence imaging on two different time points showed a decrease in metastatic tumor formation in the treatment groups compared to the controls. Immunohistochemical analysis confirmed the bioactivity of the two compounds: The Ki67 proliferation labeling index was reduced in the everolimus group and numbers of CD31 positive endothelial cells were reduced in the axitinib group.

Conclusion: Both, the mTOR inhibitor everolimus as well as antiangiogenetic effects by the VEGFR inhibitor axitinib showed potential to prevent and retard formation of symptomatic spinal metastases. However, the therapeutic efficacy was only mild in this experimental model.

Keywords: spinal metastases, targeted therapy, preclinical mouse model, everolimus, axitinib

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***Correspondence:**
Peter Vajkoczy
peter.vajkoczy@charite.de

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INTRODUCTION

Metastatic epidural spinal cord compression is a grave complication for cancer patients. If untreated, a significant spinal cord compression due to an epidural tumor mass, most frequently originating from the vertebra, will lead to relevant neurological deficits (1, 2). The metastatic process involves multiple cellular steps, such as intravasation, homing, extravasation, colony formation, and tumor growth (3). Spinal metastases occur in approximately 10 percent of cancer patients (4), they often represent the first manifestation of cancer (5), and the frequency of metastatic epidural spinal cord narrowing in cancer patients is estimated up to 8% depending on the primary tumor (6, 7). Treatment options are surgery and radiotherapy (8, 10). The gold standard in malignant extradural spinal cord compression is decompressive surgery within 24 h of diagnosis most often followed by radiotherapy (9, 10). While these treatment concepts are palliative in nature preventive therapies are not available and urgently needed.

In cancer treatment, targeted therapy has successfully developed in the last decade (11). However, an optimal pharmacological treatment specific for spinal metastatic tumors, especially in the recurrent stage or in a prophylactic setting, is not available yet. In our study, we chose two different targeted therapies, with the aim to show potential effects on spinal metastases formation and neurological outcome. Among others, inhibitors of the enzyme mammalian target of rapamycin (mTOR), as well as vascular endothelial growth factor receptor (VEGFR) inhibitors, are interesting targeting pharmaceuticals. The physiologic function of mTOR is regulating cell growth and metabolism, that is frequently highly activated in tumor cells (12), and the mTOR inhibitor and rapamycin derivate everolimus has shown promising anticancer activity in various tumor types (13, 14), with beneficial effects on bone turnover in bone metastases patients (15). Moreover, the mTOR signaling pathway is known to play a role in bone tissue signaling and in bone cancer (16). Another available targeted approach is inhibiting tumor angiogenesis, which is driven by vascular endothelial growth factor receptors (17). The tyrosine kinase inhibitor axitinib, currently approved for treatment of renal cell carcinoma (18), blocks VEGFR types 1, 2, and 3, leading to inhibition of tumor vascularization and growth (19).

In this study, we hypothesized to target the circulating tumor cell interactions with the bone vascular network by inhibiting the VEGFRs, and to block the mTOR signaling pathway to target the tumor colony formation and growth. We examined, whether targeting these signaling pathways is capable of preventing the formation of symptomatic spinal metastases in a murine hematogenous spinal metastases mouse model.

MATERIALS AND METHODS

Substances

Everolimus/RAD001 as well as axitinib (both Biozol, Eching, Germany) were solved in an injection solution consisting of 30% polyethylene glycol (PEG), 65% H_2O, 5% Tween 80, and 0.1% Dimethylsulfoxid (Sigma Aldrich, Schnelldorf, Germany). Everolimus was injected intraperitoneally (i.p.) with a concentration of 10 mg/kg body weight, and axitinib with a concentration of 25 mg/kg body weight. Placebo was the injection solution only. The i.p. injection volume was constantly 200 µl. Substances were stored at −20°C and freshly thawed immediately before use.

Firefly Luciferase Transfection, Cell Culture and Spine Metastasis-derived Cell Line Establishment

B16-F1 murine melanoma cells (ATCC® CRL-6323) were infected with a FFLUC-eGFP-Puro vector construct (B16-luc) as described previously (20). Stable cell clone growth was maintained in culture flasks stored at 37°C, 5% CO_2, as well as 95% humidity. Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, USA) was supplemented with 10% heat-inactivated fetal calf serum (Thermo Fisher Scientific, Waltham, USA), 50 units/ml penicillin and 50 µg/ml streptomycin, as well as 5 µg/ml puromycin (Sigma Aldrich, Schnelldorf, Germany) for cell selection. The mB16-luc cell line was previously established by *in vivo* selection from an already grown B16-luc spinal tumor (20).

Approval of Animal Experiments

All animal experiments were performed according to the UK Coordinating Committee on Cancer Research (UKCCR) Guidelines for the Welfare of Animals in Experimental Neoplasia (21, 22) and with the permission of the responsible local authorities from the Charité Universitätsmedizin Berlin and the LaGeSo Berlin.

Retrograde Carotid Artery Injection

We used female, 20 weeks old, C57/B6J mice (Jax Stock No. 000664). As previously described (20), they were anesthetized (9 mg ketamine hydrochlorid/1 mg xylazine per 100 g body weight) intraperitoneally and the area of the operation was shaved and kept sterile during the operation. A longitude incision of the skin was made, and under the parotid gland, the common carotid artery was prepared and the vagus nerve was separated. The artery was ligated permanently distal and temporarily proximal to the aortic arch, in between incised, and a catheter (diameter 0.8 mm, filled with 0.9% sodium chloride solution) was inserted toward the aortic arch and fixed. The temporary ligature was opened, and a 100 µl cell suspension (1×10^5 mB16-luc cells in serum-free DMEM) was slowly injected, followed by 100 µl 0.9% sodium chloride, for 1 min, respectively. Finally, the common carotid artery was completely disabled, the skin was stitched up, and mice woke up. They were randomly assigned to the placebo and the treatment groups. Despite fully common carotid artery occlusion, no neurological deficits, especially no pareses of limbs, were observed (20, 23).

Treatment Schedule and Animal Examination

All mice gained access to water and a standard laboratory diet. Mice were treated with everolimus i.p. daily for consecutive 16 days in one group, with axitinib i.p. daily for consecutive 19 days

in one group, as well as with placebo daily for consecutive 19 days in one group. Drug administration started on postoperative day 1, in order to ensure the identical tumor load in all groups at the start of therapy. Mice were examined daily, after occurrence of neurologic deficits like limb weakness, inability to run or paraplegia, or a bad health status, a spinal MRI was performed and mice were immediately sacrificed. The spine and brain were dissected, and frozen for further histological analysis.

Bioluminescence Imaging

Imaging was performed on postoperative days 11 and 22 on every mouse with the IVIS Lumina II equipment (Caliper LS, Waltham, USA). During the procedure, mice were anesthetized with 2% isoflurane (Forene, Wiesbaden, Germany), and they were shaved along the spine for better imaging. D-luciferine (Caliper LS, Waltham, USA) was administered i.p. analogous to the manufacturer's protocol (30 mg/ml, 10 µl/g body weight) in order to activate cleavage by luciferase, selectively expressed by the tumor cells. After 5 min, mice were transferred into the imaging system. After an exposure time of 5 min, signals were measured as relative light units with Living Image 3.1 software (Perkin Elmer, Waltham, USA). A demarcated area above the spine was defined as one hot spot if it showed a clear signaling and could be distinguished from other neighboring signaling areas.

Magnetic Resonance Imaging

MRI studies were performed at a seven Tesla small-animal system BioSpec 70/20 (Bruker BioSpin MRI GmbH, Ettlingen, Germany) with a BGA-12S HP gradient system and Bruker software Paravision 6.0.1. For imaging, a 1H−86 mm quadrature volume resonator and a receive—only 1H—phased array rat brain surface coil were used. During MRI examination, mice were placed on a heated circulating water blanket to ensure constant body temperature of 37°C. Anesthesia was maintained with 2.5–1.5% isoflurane delivered in an O_2/N_2O mixture (0.3/0.7 l/min) via a facemask under constant ventilation monitoring (Small Animal Monitoring & Gating System, SA Instruments, New York, USA). T2-weighted images of the whole mouse spine in the sagittal plane were made. For image acquiring, Paravision 6.0.1 software (Bruker, Billerica, USA) was used. For metastases number analysis, vertebral body as well as intraspinal tumors and tumors of the posterior column were counted.

Spine Fixation and Preparation

For fixation, a whole animal perfusion was necessary. Mice were deeply anesthetized with 9 mg ketamine hydrochlorid/1 mg xylazine per 100 g body weight i.p. A thoracotomy was performed, a needle was placed into the left ventricle, and the right atrium was opened for outflow. Perfusion began with sodium chloride, until a bright liver indicated a good effect, and was completed by infusion with 4% paraformaldehyde (24). Whole spines were dissected, muscle tissue was completely removed, and samples were fixed in 4% paraformaldehyde solution on ice for 4 h. Then, spines were decalcified with 0.5M EDTA solution at 4°C for 24 h. After that, samples were stored in 20% sucrose and 2% polyvinylpyrrolidone for 24 h at 4°C. Finally, spines were embedded in a solution of 8 g gelatin, 2 g polyvinylpyrrolidone, and 20 g sucrose in 100 ml PBS, and stored at −80°C (25).

Immunohistochemistry

Spine samples were transferred to a cryostat (Leica, Wetzlar, Germany), and cryosections were made at −20°C. Slides were dried and stored at −20 °C. For immunostaining, they were thawed and fixed in methanol (−20°C for 5 min) followed by 30 min incubation with blocking buffer (1% casein in PBS). The primary antibodies against Ki67 (Thermo Fisher Scientific, Waltham, USA) and CD31/PECAM-1 (BD Pharmingen, Franklin Lakes, USA) as well as FITC-conjugated and Cy3-conjugated secondary antibodies (Dianova, Hamburg, Germany) were added for 2 h, respectively. Cell nuclei staining was performed by incubation with 4',6-diamidino-2-phenylindole (1:100 in PBS, Roth, Karlsruhe, Germany) for 5 min. Slides were covered with Immu-Mount (Thermo Fisher Scientific, Waltham, USA). By fluorescence microscopy, 30 high-power fields from three different spinal metastases per group were randomly chosen, Ki67 positive and negative stained cell nuclei were compared by percentage with ImageJ software (NIH, Bethesda, USA), CD31 positive endothelial cells were counted and subsequently analyzed. Negative controls without processing primary antibodies did not display any specific immunoreactivity.

Statistical Analysis

Data were shown as mean ± standard deviation. The Mantel-Cox test (logrank test) was used for survival analysis as well as analysis for neurological deficit occurrence. For immunohistochemical analysis, the Kruskal–Wallis test was used to test for significant differences among groups ($p = 0.009$ for the Ki67 analysis, $p < 0.001$ for the CD31 analysis), and the Mann–Whitney U-test was applied for group comparisons. Differences with $p < 0.05$ were considered as statistically significant. Statistical differences were calculated with SPSS software (IBM, Armonk, USA) as well as with GraphPadPrism software (La Jolla, USA).

RESULTS

Spinal Epidural Metastases Development

In our mouse model, bony metastases in the spine with epidural spinal cord compression developed within 2–3 weeks, as previously shown (20, 26), without solid brain metastases formation, at least in the corresponding time periods, which could also lead to neurological deficits. Spine column explants already macroscopically showed black melanoma metastases (**Figure 1A**). Both bioluminescence imaging (**Figure 1B**) and spinal MRI (**Figure 1C**) allowed to assess spinal metastatic burden *in vivo*. Conventional histology illustrated epidural metastatic spinal cord compression and demonstrated tumors that infiltrated the trabecular vertebral bone structure and compressed the dura and spinal cord (**Figures 1D,E**).

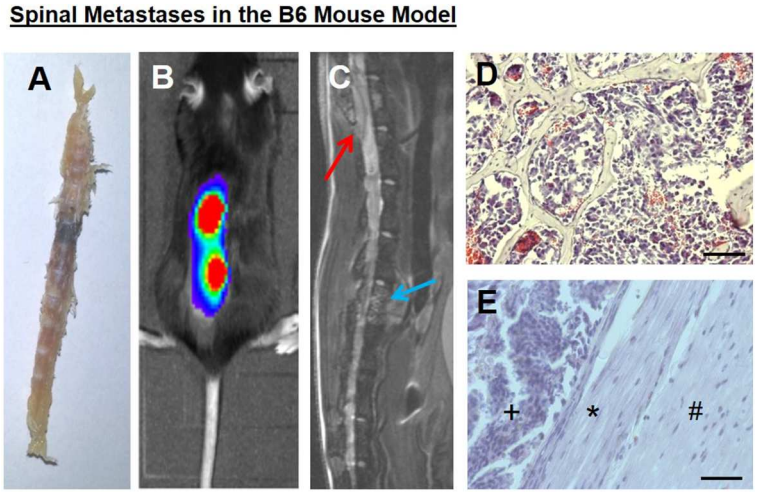


FIGURE 1 | (A) A spine column explant with a black melanoma metastasis. **(B)** Correlating to bioluminescence signals, a **(C)** spinal sagittal plane MRI shows a vertebral body metastasis, blue arrow, as well as a metastasis with epidural spinal cord compression, red arrow. **(D)** Tumor cells infiltrated the trabecular bony structure (representative hematoxylin and eosin staining). **(E)** Intraspinal epidural tumor (+), dura impression (*), nervous tissue (#). Bars indicate 100 μm.

Prolonged Overall Survival

Treatment with everolimus or axitinib prolonged overall survival of tumor bearing mice by up to 30% when compared to placebo (**Figure 2A**). In the control group, mean survival was 23 ± 4 days compared to 30 ± 8 days in the everolimus group and 28 ± 6 days in the axitinib group (**Figure 2C**). The differences compared to the placebo group were statistically significant ($p = 0.04$).

Effect on Neurological Deficit

In contrast to survival, the effect on formation of spine metastasis was less pronounced. Seventy-eight percent of mice in the control group showed paralysis of upper or lower limbs, compared to 50% in the treatment groups (**Figures 2B,C**). MRI confirmed that all of these episodes of neurological deterioration were related to a spinal metastasis. The time to development of these neurological deficits was, however, not statistically different. Mean time to neurological deficit was 22 ± 5 days in the control group. In the everolimus group, time to paresis was 26 ± 2 days ($p = 0.10$) and in the axitinib group, time to neurological deficit was 27 ± 6 days ($p = 0.06$; **Figure 2C**).

Spinal MRI Directly After Onset of Neurological Deficits

After onset of a neurological deficit, a whole spinal MRI was performed and thereafter, mice were sacrificed. The numbers of spinal metastases were counted on the spinal MRI in sagittal cuts. We found a mean metastases number of 2 ± 1 in the control group, 2 ± 3 in the everolimus group, and 2± 1 in the axitinib group, respectively ($p = 0.91$; **Figure 2C**).

Bioluminescence Tumor Visualization

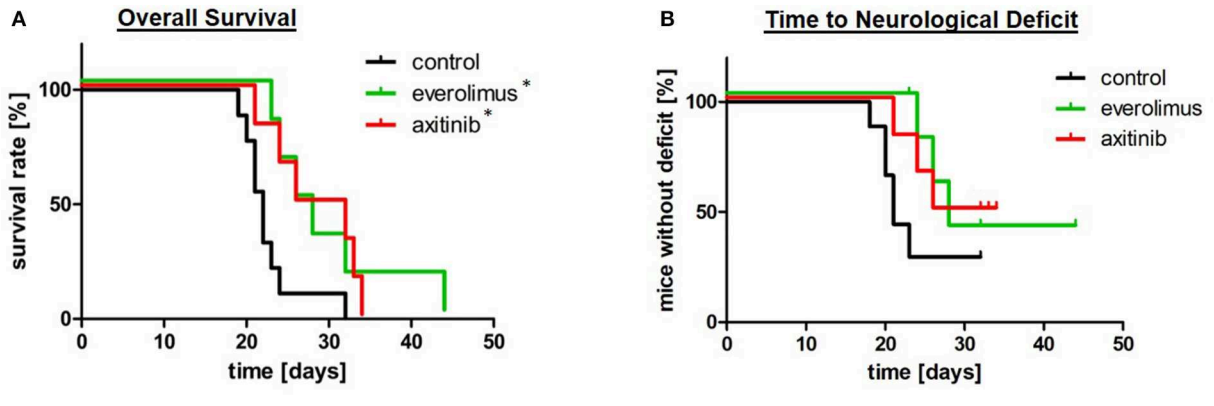
We conducted a bioluminescence spine metastatic tumor screening after postoperative days 11 and 22 (**Figure 3**). Areas of spinal hot spots correlated well with the results of MR imaging (**Figures 1B,C**). After 11 days, we observed similar numbers of metastases per mouse in the experimental group (control, everolimus and axitinib group 1 ± 1, 2 ± 1, 1 ± 1, respectively; $p > 0.05$), as well as similar numbers on postoperative day 22 (2 ± 2, 2 ± 1, 2 ± 1, respectively; $p > 0.05$). A heightened signal intensity over time of already existing metastases, indicating tumor growth, could be measured in all mice.

Histological Analysis

Next we aimed at understanding the mild effects in the treatment arms. To reveal a possible treatment effect on metastases proliferation, we examined spinal tumor tissue by Ki67 staining. In the control group, there was a mean of 22 ± 6% of stained cell nuclei, compared to 18 ± 5% in the everolimus group ($p = 0.013$), and 22 ± 5% in the axitinib group (**Figures 4A–D**). To assess anti-angiogenic activities, we stained for the endothelial cell marker CD31. There was a mean number of endothelial cells per high-power field of 25 ± 4 cells in the controls, of 25 ± 5 cells in the everolimus group, and of 19 ± 5 cells in the axitinib group. The difference between the axitinib group and the control group was statistically significant ($p < 0.001$). Furthermore, we observed larger vessels within spinal metastases of axitinib treated mice (**Figures 4E–H**).

DISCUSSION

In the present study we have shown that in our mouse model bony metastases developed within the spine causing epidural spinal cord compression, and it is possible to monitor the occurrence and numbers of spinal metastases by bioluminescence imaging or MRI. The principal novel findings are a significant prolonged overall survival of mice treated with the mTor inhibitor everolimus or after therapy with the VEGFR blocker axitinib, as well as a prolonged time to



C

	placebo group (n=9)		everolimus group (n=6)		axitinib group (n=6)	
animal no.	*day*	*event leading to death*	*day*	*event leading to death*	*day*	*event leading to death*
1	18	paraplegia lower extremities	23	cachexia, no paresis	21	paraplegia lower extremities
2	20	paraplegia lower extremities	24	paraplegia lower extremities	24	paraplegia lower extremities
3	20	paraplegia lower extremities	26	paraplegia lower extremities	26	paresis left leg
4	21	mild paresis of upper extremity, cachexia	28	paraplegia lower extremities	32	cachexia, no paresis
5	21	cachexia, no paresis	32	big hip tumor, no paresis	33	cachexia, no paresis
6	21	paraplegia lower extremities	44	dead, reason unclear	34	dead, reason unclear
7	23	paraplegia lower extremities				
8	23	cachexia, no paresis				
9	32	paresis left leg, cachexia				
mean overall survival [days]	23 ± 4		30 ± 8		28 ± 6	
mean time to neurological deficit [days]	22 ± 5		26 ± 2		27 ± 6	
paresis (upper or lower limbs)	78%		50%		50%	
number of spinal metastases in MRI (on day neurological deficit occured)	2 ± 1		2 ± 3		2 ± 1	

FIGURE 2 | Overall survival, time to neurological deficits as well as clinical symptoms. **(A)** Kaplan-Meier survival plots, comparing the control group (black), everolimus group (green), and axitinib group (red) with a significantly (*$p = 0.04$) lower mortality in the treatment groups. **(B)** Time to neurological deficit, defined by extremity paresis, with a tendency to a prolonged time to this event during everolimus or axitinib treatment, but without reaching statistical significance (everolimus group $p = 0.10$, axitinib group $p = 0.06$). **(C)** This table summarizes data of all experimental groups. The day number describes the postoperative day, when the respective event leading to death occurred.

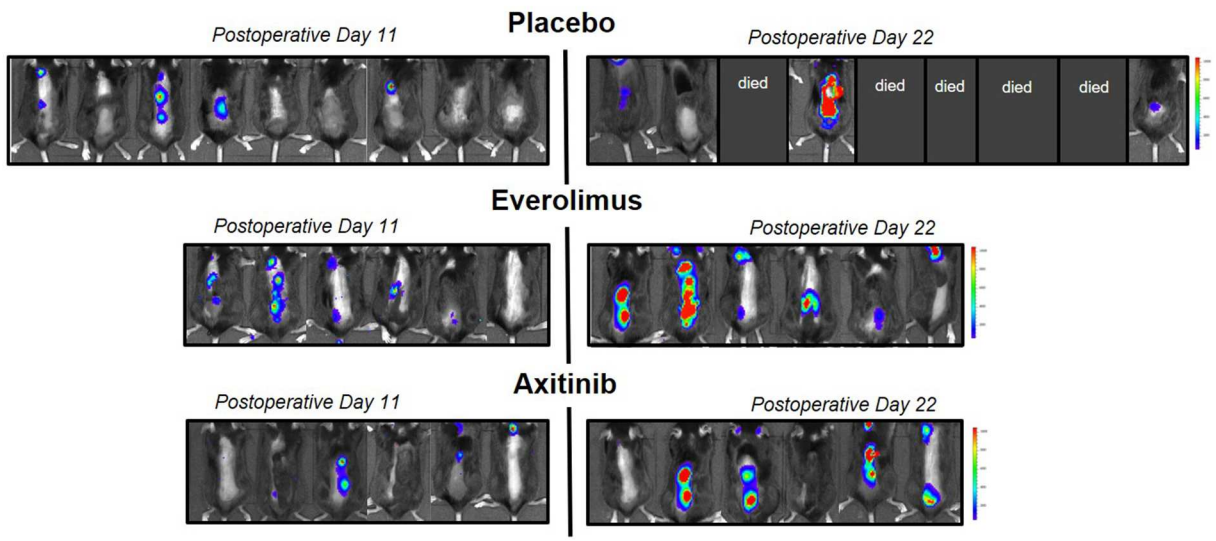
neurological deficit, triggered by a spinal metastatic tumor, in both treatment modalities.

Malignant spinal cord compression caused by spinal metastases can lead to neurological symptoms up to paraplegia with a serious reduction in quality of life. Generally, tumor metastases are located most frequently in the lungs and liver, but then followed by the skeletal system including the spine (27). Malignant spinal cord compression can indicate an advanced tumor stage, verified by the observation of a limited overall survival after diagnosis (2, 28).

In the acute situation of a neurological deficit, surgery is the therapy of choice, in order to improve or retain neurologic function. If a patient is not operable, radiotherapy combined with corticosteroids can be initiated (9). Most often, a resection is accompanied by local radiotherapy and, depending on the tumor stage, systemic chemotherapy (2). Nevertheless, the local recurrence rate after treated symptomatic spinal metastases is about 7 to 14 percent (2, 29, 30). To focus on these patients, targeted therapies as an adjunct to the standard treatment seem to be promising, with the aim to prevent as well as treat these tumors, and to stop the growth of other possibly existing metastatic tumors within the spine.

Therefore, we previously established a hematogenous spinal metastases mouse model to examine basic principles of spinal metastases formation (20). We adapted a bone metastases mouse model (31) by injecting tumor cells into the common carotid artery to avoid the pulmonary filter system, so that tumor cells spread into the arterial blood leading to an endogenously spinal metastases formation process. It is assumed that tumor cells are reaching the vertebral bodies by a hematogenous route (32), and arterial embolization seems to be the most relevant mechanism of metastasis development (33). The distribution of spinal metastases is about 60% in the thoracic, 25% in the lumbar, and 15% in the cervical spine (1, 6, 34), with similar distributions in our spinal model shown by bioluminescence monitoring, indicating a matchable system regarding to the mechanism of spinal metastases formation, which is, however, still not fully understood.

In our model, we used murine melanoma cells (35), obtaining the advantage to use immunocompetent mice, and to retain potential influences of the immune system. For cell injections, we used our stable cell line mB16, established from solid grown B16 spinal metastases in this model by *in vivo* selection, with a significantly higher potential to metastasize to the spine (20). The advantage is that mice, after tumor cell injections, generally



Mean Number of Hot Spots in Mice

	postop day 11	*postop day 22*
Placebo	1 ± 1	2 ± 2
Everolimus	2 ± 1	2 ± 1
Axitinib	1 ± 1	2 ± 1

FIGURE 3 | Metastases visualization by *in vivo* luminescence imaging. Left side shows mice on postoperative day 11, right side day 22. Blue color implies low signal intensity, red color high signal intensity, as relative light units in the color scale. In the placebo group, 5 mice already died before measurement on postoperative day 22 could be performed, in the treatment groups, all mice could be measured. A heightened signal intensity over time of metastases correlating with tumor growth could be shown in all mice.

did not suffer from significant tumor burden in other organs like the lung, liver or brain, and a nearly spine-specific tumor model was available.

One aim of our study was to confirm this preclinical model as valid for therapeutic studies regarding spinal metastases formation. Moreover, we applied two different targeted therapy approaches with the following hypothetical impacts: On the one hand, we wanted to target the potential circulating melanoma cell interactions with the bone vascular network by inhibiting the VEGFRs, in order to target the vascular formation step in the metastases origination cascade. On the other hand, we blocked the mTOR signaling pathway to potentially target tumor bone colony formations and spine metastasis growth, as the last step in metastasis bone formation.

We could find a significantly prolonged overall survival in both therapy groups. Placebo treated mice lived 23 days on average, after everolimus or axitinib therapy, mice survived 7 (prolonged by 30%) and 5 days (prolonged by 25%) longer, respectively. This indicates a general antitumor effect in the systemic compartment. In order to assess symptomatic spinal metastases, we focused on acute neurological deficits, e.g., limb paresis. A causal spinal cord compression was verified by MRI. Here, we found a 18% prolonged time after mTOR inhibition by everolimus, and a 23% prolongation after VEGFR blockade via axitinib therapy, until neurological deficits due to spinal cord compression occurred. We state that our model serves as a valid system for examining therapy responses on spinal metastases formation, and for evaluation of the neurological outcome.

The luminescence reporter imaging for tumor visualization served to detect possible dynamic effects on spinal metastases formation and growth *in vivo* (36). Between two different time points during therapy, on postoperative days 11 and 22, we did not detect any significant change in the numbers of spinal tumor hot-spots among groups. In the spinal MRI, we detected similar numbers of metastases in comparison with the bioluminescence method on postoperative day 22, considering the different time points the methods were applied. The bioluminescence screening was used on two defined time points, whereas the spinal MRI was performed directly after the occurrence of a neurological deficit in order to show the responsible spinal tumor, which apparently began on different dates for each mouse. Though, we presume a higher increase in numbers of novel developing spinal tumor hot-spots during axitinib therapy, in contrast to a continuous growth of already early existing metastases in the everolimus group. More frequent time intervals could reveal clearer effects, and being aware of the limited validity, i.a. the small differences and the small number of mice in the placebo group on the second time point, it seems possible that VEGFR inhibition could delay the time point of the development of solid spinal metastases, similar to a prophylaxis, maybe due to

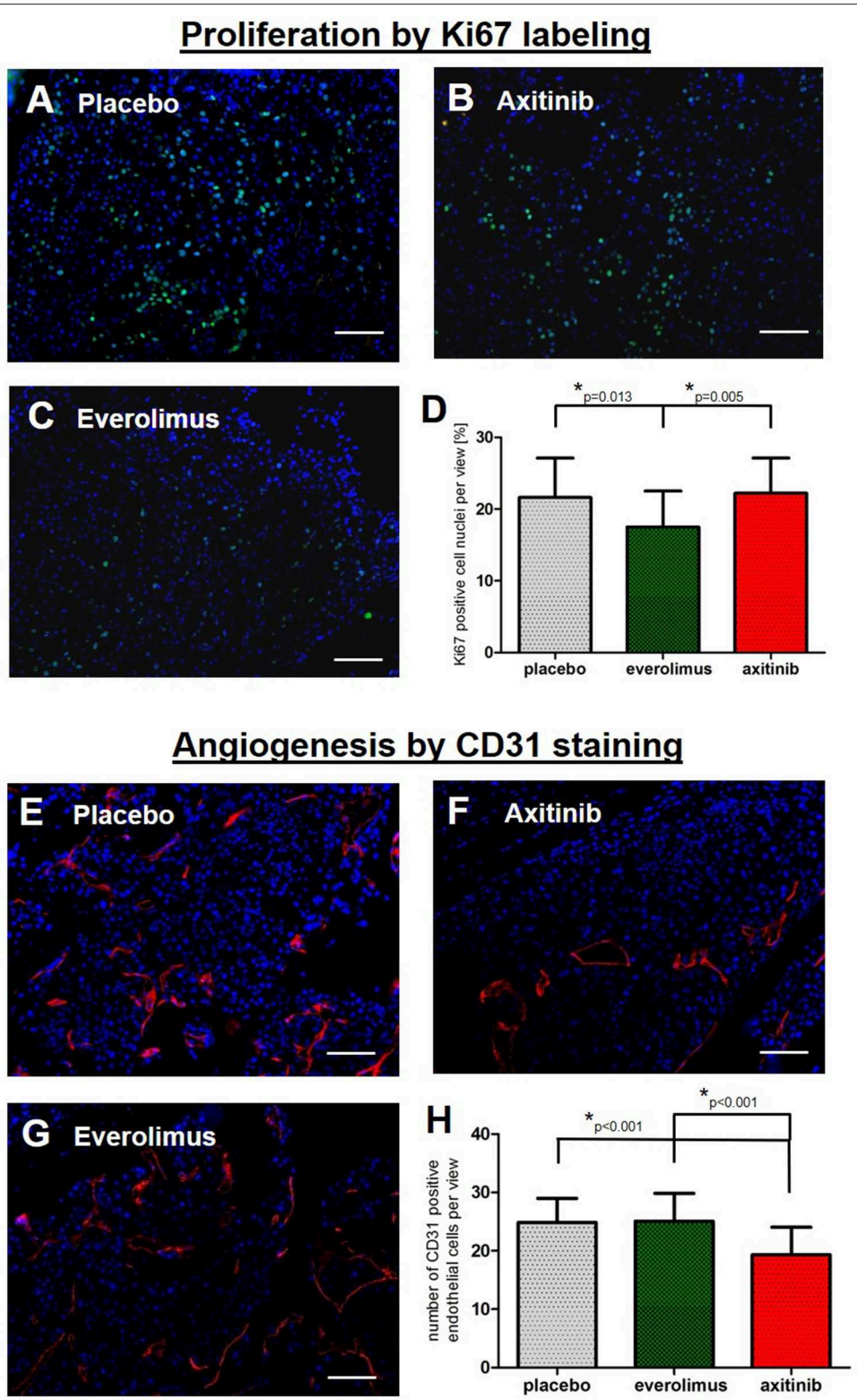


FIGURE 4 | Immunohistochemical analysis. Graphs show the summary of data from all randomly chosen high-power fields from 3 different spinal metastases per group. **(A–D)** Fluorescence-provided Ki67 stainings of tumor cell nuclei in the placebo group, axitinib group and everolimus group. The fraction of proliferating cells was lowered in the everolimus group ($p = 0.013$). **(E–H)** Numbers of endothelial cells marked by CD31 immunostainings revealing a lowered number of endothelial cells as well as larger tumor vessels in axitinib treated mice ($p < 0.001$).

angiogenesis inhibition after tumor cells are entrapped passively (26). For example, in prostate tumors, the expression levels of VEGF and VEGFRs were higher at the bone metastases site compared to the primary tumors, indicating the importance of angiogenesis in metastasis development to the bone (37), and vascular factors could encourage the nesting of tumor cells in the bone (38). Angiogenesis inhibition could be revoked by tumor cell adaptation processes, and the antiangiogenic effects could be pronounced in early stages of metastases formation. At this time point, tumors could be most accessible to targeted VEGFR inhibition, with the focus on vessel formation, delaying the process of building their own blood supply. This theory could be addressed in future projects.

Further immunohistochemical analysis showed that in late stage spinal metastases, the proliferation of tumor cells was slightly lowered after mTOR inhibition, detected by a decrease in the Ki67 labeling index. VEGFR inhibition did not influence the tumor proliferation rate, but reduced the number of endothelial cells, and larger vessels were observed, indicating a normalization of the tumor vasculature, and possible antiangiogenic effects. This indicated two different impacts on malignant tumor properties after two different targeted treatments against spinal metastases, also confirming the bioactivity of the two applied compounds. Here, the mild histological effects could not finally confirm the expected mode of actions as a therapy target, and our applied therapy did not elicit a significant impact on the spinal tumor compartment, though proved significant prolongation of the overall survival as well as prolongation of the occurrence of neurological deficits.

In this study, we demonstrated a feasible preclinical mouse model suitable for investigating targeted therapy approaches against metastatic spinal cord compression. It is important to better understand the molecular mechanisms in spinal metastases formation and further experimental data is necessary, with the general aim to offer patients individualized therapy options.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Landesamt für Gesundheit und Soziales (LAGeSo) Berlin, Turmstraße 21, 10559 Berlin, Germany.

AUTHOR CONTRIBUTIONS

PV and TK designed the study. TK carried out and analyzed the experiments and wrote the manuscript. AP, TB, and MC gave support to experiments. PV and TK revised the manuscript. All authors approved the final version.

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REFERENCES

1. Bartels RH, van der Linden YM, van der Graaf WT. Spinal extradural metastasis: review of current treatment options. *CA Cancer J Clin.* (2008) 58:245–59. doi: 10.3322/CA.2007.0016
2. Cole JS, Patchell RA. Metastatic epidural spinal cord compression. *Lancet Neurol.* (2008) 7:459–66. doi: 10.1016/S1474-4422(08)70089-9
3. Valastyan S, Weinberg RA. Tumor metastasis: molecular insights and evolving paradigms. *Cell.* (2011) 147:275–92. doi: 10.1016/j.cell.2011.09.024
4. Sciubba DM, Petteys RJ, Dekutoski MB, Fisher CG, Fehlings MG, Ondra SL, et al. Diagnosis and management of metastatic spine disease. A review. *J Neurosurg. Spine.* (2010) 13:94–108. doi: 10.3171/2010.3.SPINE09202
5. Schiff D, O'Neill BP, Suman VJ. Spinal epidural metastasis as the initial manifestation of malignancy: clinical features and diagnostic approach. *Neurology.* (1997) 49:452–6. doi: 10.1212/WNL.49.2.452
6. Bach F, Larsen BH, Rohde K, Børgesen SE, Gjerris F, Bøge-Rasmussen T, et al. Metastatic spinal cord compression. Occurrence, symptoms, clinical presentations and prognosis in 398 patients with spinal cord compression. *Acta Neurochir.* (1990) 107:37–43. doi: 10.1007/BF01402610
7. Loblaw DA, Laperriere NJ, Mackillop WJ. A population-based study of malignant spinal cord compression in Ontario. *Clin Oncol* (*R Coll Radiol*). (2003) 15:211–7. doi: 10.1016/S0936-6555(02)00400-4
8. Fehlings MG, Nater A, Tetreault L, Kopjar B, Arnold P, Dekutoski M, et al. Survival and clinical outcomes in surgically treated patients with metastatic epidural spinal cord compression: results of the prospective multicenter aospine study. *J Clin Oncol.* (2016) 34:268–76. doi: 10.1200/JCO.2015.61.9338
9. Loblaw DA, Mitera G, Ford M, Laperriere NJ. A 2011 updated systematic review and clinical practice guideline for the management of malignant extradural spinal cord compression. *Int J Radiat Oncol Biol Phys.* (2012) 84:312–7. doi: 10.1016/j.ijrobp.2012.01.014
10. Patchell RA, Tibbs PA, Regine WF, Payne R, Saris S, Kryscio RJ, et al. Direct decompressive surgical resection in the treatment of spinal cord compression caused by metastatic cancer: a randomised trial. *Lancet.* (2005) 366:643–48. doi: 10.1016/S0140-6736(05)66954-1
11. Brown C. Targeted therapy: an elusive cancer target. *Nature.* (2016) 537:S106–8. doi: 10.1038/537S106a
12. Brachmann S, Fritsch C, Maira SM, Garcia-Echeverria C. PI3K and mTOR inhibitors: a new generation of targeted anticancer agents. *Curr Opin Cell Biol.* (2009) 21:194–8. doi: 10.1016/j.ceb.2008.12.011
13. Krueger DA, Care MM, Holland K, Tudor C, Mangeshkar P, Wilson KA, et al. Everolimus for subependymal giant-cell astrocytomas in tuberous sclerosis. *N Engl J Med.* (2010) 363:1801–11. doi: 10.1056/NEJMoa1001671
14. Yao JC, Shah MH, Ito T, Bohas CL, Wolin EM, Cutsem EV, et al. Everolimus for advanced pancreatic neuroendocrine tumors. *N Engl J Med.* (2011) 364:514–23. doi: 10.1056/NEJMoa1009290
15. Gnant M, Baselga J, Rugo HS, Noguchi S, Burris HA, Piccart M, et al. Effect of everolimus on bone marker levels and progressive disease in bone in BOLERO-2. *J Natl Cancer Inst.* (2013) 105:654–63. doi: 10.1093/jnci/djt026

16. Bertoldo F, Silvestris F, Ibrahim T, Cognetti F, Generali D, Ripamonti CI, et al. Targeting bone metastatic cancer: role of the mTOR pathway. *Biochim Biophys Acta.* (2014) 1845:248–54. doi: 10.1016/j.bbcan.2014.01.009
17. Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L. VEGF receptor signalling - in control of vascular function. *Nat Rev Mol Cell Biol.* (2006) 7:359–71. doi: 10.1038/nrm1911
18. Rixe O, Bukowski RM, Michaelson MD, Wilding G, Hudes GR, Bolte O, et al. Axitinib treatment in patients with cytokine-refractory metastatic renal-cell cancer: a phase II study. *Lancet Oncol.* (2007) 8:975–84. doi: 10.1016/S1470-2045(07)70285-1
19. Hu-Lowe DD, Zou HY, Grazzini ML, Hallin ME, Wickman GR, Amundson K, et al. Nonclinical antiangiogenesis and antitumor activities of axitinib (AG-013736), an oral, potent, and selective inhibitor of vascular endothelial growth factor receptor tyrosine kinases 1, 2, 3. *Clin Cancer Res.* (2008) 14:7272–83. doi: 10.1158/1078-0432.CCR-08-0652
20. Broggini T, Czabanka M, Piffko A, Harms C, Hoffmann C, Mrowka R, et al. ICAM1 depletion reduces spinal metastasis formation *in vivo* and improves neurological outcome. *Eur Spine J.* (2015) 24:2173–81. doi: 10.1007/s00586-015-3811-7
21. UKCCCR. United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) Guidelines for the Welfare of Animals in Experimental Neoplasia (Second Edition). *Br J Cancer.* (1998) 77:1–10. doi: 10.1038/bjc.1998.1
22. Workman P, Aboagye EO, Balkwill F, Balmain A, Bruder G, Chaplin DJ, et al. Guidelines for the welfare and use of animals in cancer research. *Br J Cancer.* (2010) 102:1555–77. doi: 10.1038/sj.bjc.6605642
23. Kitagawa K, Yagita Y, Sasaki T, Sugiura S, Omura-Matsuoka E, Mabuchi T, et al. Chronic mild reduction of cerebral perfusion pressure induces ischemic tolerance in focal cerebral ischemia. *Stroke.* (2005) 36:2270–4. doi: 10.1161/01.STR.0000181075.77897.0e
24. Gage GJ, Kipke DR, Shain W. Whole animal perfusion fixation for rodents. *J Vis Exp.* (2012) 65:3564. doi: 10.3791/3564
25. Kusumbe AP, Ramasamy SK, Starsichova A, Adams RH. Sample preparation for high-resolution 3D confocal imaging of mouse skeletal tissue. *Nat Protoc.* (2015) 10:1904–14. doi: 10.1038/nprot.2015.125
26. Broggini T, Piffko A, Hoffmann CJ, Harms C, Vajkoczy P, Czabanka M. Passive entrapment of tumor cells determines metastatic dissemination to spinal bone and other osseous tissues. *PLoS ONE.* (2016) 11:e0162540. doi: 10.1371/journal.pone.0162540
27. Witham TF, Khavkin YA, Gallia GL, Wolinsky JP, Gokaslan ZL. Surgery insight: current management of epidural spinal cord compression from metastatic spine disease. *Nat Clin Pract Neurol.* (2006) 2:87–94. doi: 10.1038/ncpneuro0116
28. Rades D, Fehlauer F, Schulte R, Veninga T, Stalpers LJA, Basic H, et al. Prognostic factors for local control and survival after radiotherapy of metastatic spinal cord compression. *J Clin Oncol.* (2006) 24:3388–93. doi: 10.1200/JCO.2005.05.0542
29. van der Sande JJ, Boogerd W, Kroger R, Kappelle AC. Recurrent spinal epidural metastases: a prospective study with a complete follow up. *J Neurol Neurosurg Psychiatry.* (1999) 66:623–7. doi: 10.1136/jnnp.66.5.623
30. Chamberlain MC, Kormanik PA. Epidural spinal cord compression: a single institution's retrospective experience. *Neuro Oncol.* (1999) 1:120–3. doi: 10.1093/neuonc/1.2.120
31. Arguello F, Baggs RB, Frantz CN. A murine model of experimental metastasis to bone and bone marrow. *Cancer Res.* (1988) 48:6876–81.
32. Algra PR, Heimans JJ, Valk J, Nauta JJ, Lachniet M, Van Kooten B. Do metastases in vertebrae begin in the body or the pedicles? Imaging study in 45 patients. *AJR Am J Roentgenol.* (1992) 158:1275–9. doi: 10.2214/ajr.158.6.1590123
33. Arguello F, Baggs RB, Duerst RE, Johnstone L, McQueen K, Frantz CN. Pathogenesis of vertebral metastasis and epidural spinal cord compression. *Cancer.* (1990) 65:98–106. doi: 10.1002/1097-0142(19900101)65:1<98::aid-cncr2820650121>3.0.co;2-k
34. Constans JP, de Divitiis E, Donzelli R, Spaziante R, Meder JF, Haye C. Spinal metastases with neurological manifestations. Review of 600 cases. *J Neurosurg.* (1983) 59:111–8. doi: 10.3171/jns.1983.59.1.0111
35. Fidler IJ. Selection of successive tumour lines for metastasis. *Nat N Biol.* (1973) 242:148–9. doi: 10.1038/newbio242148a0
36. Rehemtulla A, Stegman LD, Cardozo SJ, Gupta S, Hall DE, Contag CH, et al. Rapid and quantitative assessment of cancer treatment response using *in vivo* bioluminescence imaging. *Neoplasia.* (2000) 2:491–5. doi: 10.1038/sj.neo.7900121
37. Chen J, De S, Brainard J, Byzova TV. Metastatic properties of prostate cancer cells are controlled by VEGF. *Cell Commun Adhes.* (2004) 11:1–11. doi: 10.1080/15419060490471739
38. Sterling JA, Edwards JR, Martin TJ, Mundy GR. Advances in the biology of bone metastasis: how the skeleton affects tumor behavior. *Bone.* (2011) 48:6–15. doi: 10.1016/j.bone.2010.07.015

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REVIEW
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Calcium Homeostasis: A Potential Vicious Cycle of Bone Metastasis in Breast Cancers

Zhengfeng Yang[1*†], *Zhiying Yue*[2†], *Xinrun Ma*[1] *and Zhenyao Xu*[1]

[1] *Shanghai Institute of Immunology Center for Microbiota & Immune Related Diseases, Institute of Translational Medicine, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China,* [2] *Department of Urology, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China*

Cancers have been considered as one of the most severe health problems in the world. Efforts to elucidate the cancer progression reveal the importance of bone metastasis for tumor malignancy, one of the leading causes for high mortality rate. Multiple cancers develop bone metastasis, from which breast cancers exhibit the highest rate and have been well-recognized. Numerous cells and environmental factors have been believed to synergistically facilitate bone metastasis in breast cancers, from which breast cancer cells, osteoclasts, osteoblasts, and their produced cytokines have been well-recognized to form a vicious cycle that aggravates tumor malignancy. Except the cytokines or chemokines, calcium ions are another element largely released from bones during bone metastasis that leads to hypercalcemia, however, have not been well-characterized yet in modulation of bone metastasis. Calcium ions act as a type of unique second messenger that exhibits omnipotent functions in numerous cells, including tumor cells, osteoclasts, and osteoblasts. Calcium ions cannot be produced in the cells and are dynamically fluxed among extracellular calcium pools, intracellular calcium storages and cytosolic calcium signals, namely calcium homeostasis, raising a possibility that calcium ions released from bone during bone metastasis would further enhance bone metastasis and aggravate tumor progression via the vicious cycle due to abnormal calcium homeostasis in breast cancer cells, osteoclasts and osteoblasts. TRPs, VGCCs, SOCE, and P2Xs are four major calcium channels/routes mediating extracellular calcium entry and affect calcium homeostasis. Here we will summarize the overall functions of these four calcium channels in breast cancer cells, osteoclasts and osteoblasts, providing evidence of calcium homeostasis as a vicious cycle in modulation of bone metastasis in breast cancers.

Keywords: calcium homeostasis, calcium channels, vicious cycle, tumor progression, osteoclast activation, bone metastasis

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***Correspondence:**
Zhengfeng Yang
zhengfeng.yang@shgh.cn

† These authors have contributed equally to this work

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INTRODUCTION

Cancers have been considered as a worldwide health problem for years. In 2018, 18 million new cases of cancers were diagnosed and around 9.6 million death was reported, which accounts for the second leading cause of death in the world (1). Cancer metastasis has been well-recognized as one of the major causes for cancer progression as well as the high cancer mortality rate, especially the bone metastasis (2). Multiple type of cancers develop bone metastasis, including breast, prostate, thyroid,

lung, renal, melanoma, head and neck, gastrointestinal tract and ovarian, from which breast cancer and prostate cancer are two typical types that exhibit highest percentage of bone metastasis rate, with ∼70% in both breast cancers and prostate cancers (3).

Bone metastasis is a process by which primary tumor cells spread to the bone through the bloodstream or lymph vessel. The migrated/metastasized tumor cells then proliferate in the bone and enhance abnormal osteoclastogenesis or osteoblastogenesis (3). In breast cancers, bone metastasis promotes osteoclast activation and leads to over-activated osteolysis. The osteolytic lesions provide comfortable niches that multiple cells including tumor cells, osteoblasts, and osteoclasts communicating with each other, resulting in continuous tumor growth in the bone as well as in the primary sites that forms a vicious cycle (4). The vicious cycle is frequently observed in the late-stage of breast cancers (stage IV) and is a multi-step processes require numerous types of cells to participate in. Briefly, breast cancer cells in the primary sites invade in the surrounded blood vessels probably via the epithelial–mesenchymal transition (5). The infiltrated tumor cells survive in the vessel with interacting with host cells, including red blood cells, neutrophils, platelets, etc., and migrate to different sites for organ invasion. Bone metastasis is occurred when the tumor cells in the vessel migrate to the bone, where the tumor cells undergo mesenchymal-epithelial transition (6, 7). Also, neovascularization is accompanied with bone metastasis, which the endothelial cells could be activated by angiogenic factors secreted by tumor cells and bone marrow (8, 9). This vasculogenesis has also been believed to facilitate bone metastasis of breast cancer cells (10). Other cells, include adipocytes, myeloid cells and Treg, all have been shown to promote bone metastasis via direct cell-cell contacts or indirect secretion of cytokines.

Numerous chemokines or cytokines facilitate bone metastasis through either the autocrine or the paracrine pattern, which have been well-summarized in these two decades (4, 11–13). Recently, calcium ions have also been reported to greatly modulate cancer progression (14, 15), however, have not been well-summarized yet. Calcium ions are one of the important second messengers that decode extracellular stimulation and thus regulate biological functions (15, 16). Unlike other second messengers, calcium ions are not produced by cell itself, but all come from extracellular calcium entry, which forms calcium homeostasis in a cell (17). Calcium homeostasis is largely affected during bone metastasis as bone is the major organ for calcium storage. Briefly, the abnormally enhanced osteoclastogenesis in cancer patients would increase bone resorption and lead to huge amounts of calcium release into blood as 99% calcium is stored in the bone (4, 18). The released calcium would then further affect bone metastasis via the abnormal calcium homeostasis in tumor cells, osteoclasts or osteoblasts, forming another potential vicious cycle for bone metastasis (**Figure 1**). Indeed, cancer patients have been observed to suffer hypercalcemia (19). Importantly, the survival rate of cancer patients with hypercalcemia is largely reduced (19), specifically the 1-year survival rate is below 30%, indicating the importance of the blood calcium levels in modulating cancer progression. These clinical observations raise an important question that how hypercalcemia worsens the progression of tumors, by targeting tumor cells, osteoclasts, osteoblasts, or the communication niches? Here we will take the breast cancer as the typical representative to specifically overview recent efforts on understanding the functions and mechanism of calcium homeostasis in breast cancer cells, osteoclasts, osteoblasts and thus bone metastasis.

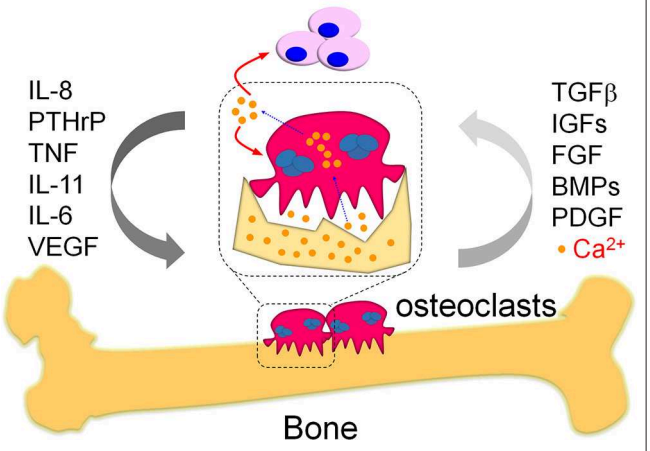

FIGURE 1 | The vicious cycle in bone metastasis. Bone metastasis is highly occurred in breast cancer patients, which results in vicious cycles to further deteriorate the primary tumor burden and promote tumor growth in bone. One kind of the well-known vicious cycles is a systemic response in tumor cells and osteoclasts. Briefly, the migrated tumor cells produce multiple cytokines and eventually enhance osteoclast differentiation as indicated. The pathologically activated osteoclasts heavily destroy bone that release numerous growth factors from damaged bone matrix, which would in turn promote tumor cell growth and therefore form the vicious cycle between tumor cells and osteoclasts during bone metastasis. Except growth factors, the damaged bone also releases large amounts of calcium ions. These calcium ions were specifically released in the sealing zones where mature osteoclasts tightly attached with the bone. The mature osteoclasts also exhibit specialized plasma membrane in the sealing zone, named as ruffled border. The ruffled border facilitate the release of calcium ions from bone via the vesicular transport in osteoclasts. The released calcium ions might further aggravate bone metastasis via modulation of the activity in tumor cells and osteoclasts, which is a potential vicious cycle required further efforts to elucidate.

CALCIUM HOMEOSTASIS IN THE CELLS

Calcium ions are well-characterized as the second messenger for multiple signaling pathways that modulate numerous biological functions, including muscle contraction, apoptosis, neural transmission, cell differentiation, and cell metabolism, etc. (16, 20), which is largely dependent on its hundreds of patterns, such as calcium release, calcium oscillations, calcium spikes, etc. These patterns are the result of calcium fluxes among cytosolic calcium ions, intracellular calcium storages and extracellular calcium pools. Normally, the extracellular calcium pool is maintained at the concentration of 1–2 mM, and the calcium level in the intracellular calcium storage, specifically the endoplasmic reticulum (ER), is around 100–400 μM. Whereas, the cytosolic calcium level is about 100 nM (17). Such gradient

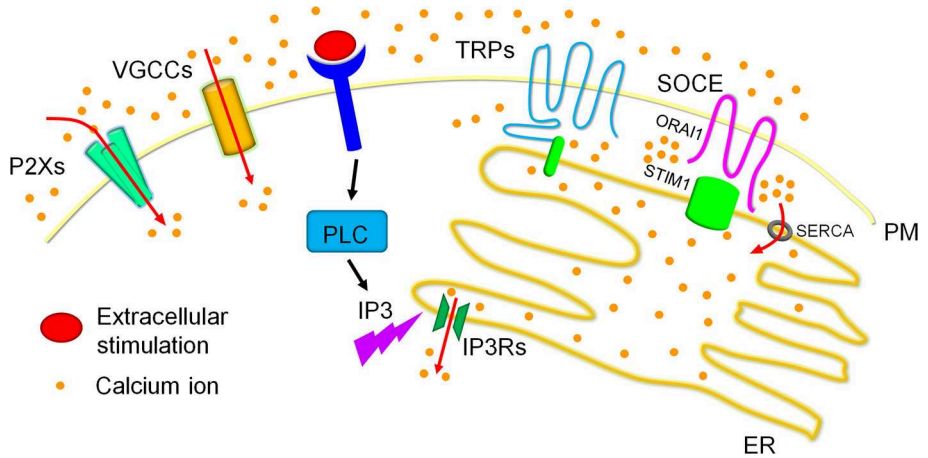

FIGURE 2 | Calcium homeostasis in cells. Calcium ions are dynamically fluxed among extracellular calcium pools, intracellular calcium storages and the cytoplasmic matrix. Four major calcium channels/routes mostly on the plasma membrane (PM) mediate extracellular calcium entry, including P2Xs, VGCCs, TRPs, and SOCE, of which TRPs and SOCE have been reported to modulate dynamic calcium fluxes in response to the reduction of calcium concentrations in the ER calcium storage. Particularly, extracellular stimulation activated PLC family members result in the production of IP3. The produced IP3 swiftly binds to IP3Rs on the ER membrane and triggers ER calcium release, which leads to the reduction of ER calcium storage. The reduced ER calcium storage activates calcium release-activated channels (CRAC) for extracellular calcium entry to refill the ER calcium ions and therefore sustain cytosolic calcium signals. TRPs especially TRPCs and SOCE have been reported to mediate CRAC. TRPCs have been shown to associate with STIM1 on the ER membrane to generate the channel for calcium entry. SOCE, the best recognized route for CRAC in recent decade, is mediated by ORAI1 on the PM and STIM1 on the ER membrane. Briefly, STIM1 is inactivated when calcium concentrations are maintained highly in the ER calcium storage, and underwent conformational change after the reduction of ER calcium contents, specifically initiated after ∼25% reduction. The conformational changed STIM1 are then oligomerized and redistributed to the ER-PM contact to interact with the clustered ORAI1, forming the calcium channel for extracellular calcium entry. These entered calcium ions can enter the ER calcium storage via the SERCA on the ER membrane.

makes the calcium signaling be dynamically modulated without hurting the cell viability that would be affected by abnormal calcium levels in the cytoplasm. Indeed, the half-life of IP3, the major messenger inducing calcium signals, is 60 s (21), a pretty short period sufficiently activating calcium release from ER, transducing downstream signals, but not abnormally increasing the cytosolic calcium level that would be toxic to the cells.

Particularly, calcium fluxes could be initiated by extracellular stimulators, such as G-protein coupled receptors and their ligands. The activated signals were then transduced to promote IP3 production via PLC family members. IP3 can bind to its receptors IP3Rs on the ER and therefore induces calcium release from ER (22) (**Figure 2**). These released calcium activates downstream signals by binding to the targeting proteins, like Ca^{2+} /calmodulin-dependent protein kinase (CAMK) and calcineurin, and eventually induces the nuclear translocation as well as the transcriptional activity of NFAT family members (23). Importantly, the calcium-NFAT axis is specifically modulated in particular cell types (24, 25), like T cells and osteoclasts, where calcium release is continuously occurred and maintained with a high frequency but a low amplitude, namely calcium oscillations, a more efficient way to facilitate NFAT activation.

Calcium oscillations are one of the typical representatives of calcium homeostasis in a cell. For instance, in osteoclasts the RANKL/RANK signaling recruits TRAF6 and activates PLCγ2 (26, 27), after which the phosphatidylinositol biphosphate (PIP2) is converted into IP3 and DAG. The produced IP3 then bind to IP3Rs as mentioned above, triggering calcium release from ER that initiates the calcium signal but also reduces the ER calcium storage. To sustain the calcium oscillations, the reduced calcium storage then promotes the activation of STIM1 located in the ER membrane that induces extracellular calcium entry by association with ORAI1 on the plasma membrane, which refills the reduced or depleted ER calcium storage and facilitates further ER calcium release (28–31) (**Figure 2**). The continued cycles of these calcium fluxes form calcium oscillations and sustain the NFAT activation (24).

Though STIM1/ORAI1 mediated calcium entry is one of the most important routes for extracellular calcium entry in non-excitable cells reported in a decade, multiple calcium channels are responsible for extracellular calcium entry, mainly including four major families. The dynamic calcium levels modulated by these channels have been reported to be critical signals for cancer cells viability and tumor formation (**Table 1**). In the following parts, we will summarize how these calcium channels modulate tumor progression and their potency in regulation of bone metastasis in breast cancers.

TRANSIENT RECEPTOR POTENTIAL (TRP) CHANNELS

The TRP channels are six transmembrane channels located mostly on the plasma membrane and sense multiple physiological stimuli, including vision, taste, olfaction, hearing, touch, thermo- and osmo-sensation (96). In response to these stimuli, the TRPs act as cation channels for multiple ions, from which calcium is one of the best recognized ions (97). The mammalian

TABLE 1 | Mammalian calcium channels and their functions in breast cancer cells and osteoclasts.

Family	Members	Functions in breast cancer cells	Functions in osteoclasts	References
TRPC	TRPC1	Proliferation ↑ TGFβ-induced EMT↑	I-mfa deficient osteoclast differentiation↑	(32–36)
	TRPC2	/	/	/
	TRPC3	/	/	/
	TRPC4	/	/	/
	TRPC5	Drug resistance ↑ Angiogenesis ↑	/	(37–39)
	TRPC6	Proliferation, survival and migration ↑	/	(40)
	TRPC7	/	/	/
TRPV	TRPV1	Drug resistance ↓ metastatic bone pain ↑	Osteoclast differentiation and activation ↑	(41–44) (45, 46)
	TRPV2	/	Calcium oscillations and osteoclastogenesis ↑	(47, 48)
	TRPV3	/	/	/
	TRPV4	Apoptosis and oncosis ↑ Actin reorganization and tumor invasion ↑	Late-stage osteoclast activation ↑	(49–51) (52–54)
	TRPV5	/	Size and number ↓ Bone resorption ↑	(55, 56)
	TRPV6	Proliferation ↑ Drug resistance ↓	Size and number ↓ Bone resorption ↓	(57–59) (60)
TRPM	TRPM1	/	/	/
	TRPM2	Cell viability ↑	/	(61)
	TRPM3	/	/	/
	TRPM4	/	/	/
	TRPM5	/	/	/
	TRPM6	/	/	/
	TRPM7	Metastasis ↑ Mesenchymal feature ↑ Proliferation ↑	/	(62–65)
	TRPM8	EMT and migration ↑	/	(66)
TRPA	TRPA1	Apoptosis ↓ Drug resistance ↑	/	(67)
TRPP	TRPP2	Drug resistance ↑	/	(68)
	TRPP3	/	/	/
	TRPP5	/	/	/
TRPML	TRPML1	Tumor growth and migration ↑	Lysosomal functions and osteoclast activation ↑	(69, 70)
	TRPML2	/	/	/
	TRPML3	/	/	/
VGCC	Cav1.1	/	/	/
	Cav1.2	/	/	/
	Cav1.3	Proliferation ↑	/	(71)
	Cav1.4	/	/	/
	Cav2.1	/	/	/
	Cav2.2	/	/	/
	Cav2.3	/	/	/
	Cav3.1	Proliferation ↓ Apoptosis ↑	/	(72)
	Cav3.2	Proliferation ↑	/	(73)
	Cav3.3	Proliferation ↑	/	(74)
SOCE	STIM1	Migration and metastasis ↑ EMT↑	Calcium oscillations ↑	(75–80)
	ORAI1	Focal adhesion, migration and invasion ↑ Bone metastasis ↑	Fusion and differentiation ↑	(76, 81–84)
P2X	P2X1	/	/	/
	P2X2	/	/	/
	P2X3	/	/	/
	P2X4	/	/	/
	P2X5	/	/	/
	P2X6	/	/	/
	P2X7	Proliferation ↑ Apoptosis ↓ Migration, metastasis ↑	Fusion and differentiation in pathological conditions ↑	(85–89) (90–95)

↑, *indicates the functions have been upregulated;* ↓, *indicates the functions have been downregulated.*

TRPs are a large superfamily that contains six subfamilies and around 30 members, including TRPCs, TPRVs, TRPMs, TRPA1, TRPPs, and TRPMLs (96, 98), which leads to a diverse cation selectivity in multiple cells, from neuron to non-neuron cells. Though TRPs exhibit multiple activation patterns, they share one common mechanism coupled to phospholipase C (PLC) activation and are responsible for extracellular calcium entry. For instance, activation of PLC by upstream signals like G-protein couple receptors would lead to calcium release from ER, the reduced calcium in the ER would then activate TRPs for extracellular calcium influx, similar as the store-operated calcium entry, to sustain further calcium signals in the cells (97). The exact mechanism linked PLC and TRPs activation is still not fully clarified and required to be discussed case by case, which has been well-described elsewhere (96, 99). Following we will summarize recent advances in understanding the function of TRPs in regulation of tumor progression and bone metastasis.

TRPCs

TRPCs in Breast Cancers

The mammalian TRPCs contain seven members, from TRPC1 to TRPC7, from which human TRPCs contain six members

as human TRPC2 is a pseudogene. TRPCs have been believed to promote tumor cell proliferation and survival in multiple tumor cells, including colon cancers, non-small cell lung cancers, glioma, gastric and esophageal cancers (100). Importantly, TRPCs have been shown to have broad functions during breast tumor progression. TRPCs have been shown to express in multiple solid tumors. In breast cancers, TRPC1 and TRPC6 are found to be highly expressed in human breast ductal adenocarcinoma compared to the adjacent non-tumor tissues (32, 101, 102), indicating the potential roles of these two TRPCs in modulation of breast tumor progression. Several studies found that TRPC1 promotes breast cancer cell proliferation and facilitates TGFβ-induced epithelial-mesenchymal transition (EMT) (32–35), suggesting that TRPC1 is an essential signal for breast tumor growth and metastasis. Indeed, TRPC1 is expressed highly in basal breast cancer cell lines and tumor tissues from patients suffering basal breast cancers, especially those accompanied with lymph node metastasis. Mechanistically, TRPC1 is required for AKT activation to induce HIF1α expression, and thus promotes EMT. Similarly, TRPC6 is also highly expressed in breast cancer cell lines compared to normal control. Silencing TRPC6 largely reduces proliferation, survival and migration in breast cancer cell lines (40), which might be due to reduced expression of ORAI1 and ORAI3 in TRPC6 deficient cells. TRPC5 is another TRPC that has been well-addressed in breast cancers progression. Unlike TRPC1 and TRPC6, TRPC5 has been identified to mediate chemotherapeutic resistance in breast cancers. When breast cancer cells or patients are treated with adriamycin, TRPC5 is upregulated in extracellular vesicles, which is believed to be responsible for the drug resistance (37, 38). Further studies show that TRPC5 also mediates autophagy by the CaMKKβ/AMPKα/mTOR pathway and therefore enhances the adriamycin resistance in breast cancers (39). In addition, TRPC5 is also upregulated in breast cancers and mediates angiogenesis during tumor progression, which is another important aspect that TRPC5 promotes breast cancers metastasis. So far, no more other TRPCs have been reported to affect breast tumor formation and development. TRPCs could be classified as four subsets according to their amino acids similarity, including TRPC1, TRPC2, TRPC3/6/7, and TRPC4/5 (96). Considering that TRPC1, TRPC5, and TRPC6, representatives of the different three subsets, all mediate calcium influx and are required for tumor cells proliferation and metastasis in breast cancer cells, the other TRPCs, specifically TRPC3, TRPC4, and TRPC7, might be also important modulators of breast cancer progression in certain scenarios. Further studies are required to elucidate the expression profiles of these TRPCs in different stages of breast cancers, which might give evidence to elucidate how these TRPCs independently or synergistically modulate breast cancers progression.

TRPCs in Osteoclasts

Till now little has been known about TRPCs in regulation of osteoclastogenesis except TRPC1 (36). TRPC1 knockout mice exhibit normal osteoclastogenesis and bone mass in physiological conditions. However, deficiency of I-mfa, the inhibitor of Trpc1, increases osteoclast differentiation and reduces bone mass. Importantly, I-mfa and Trpc1 double knockout mice exhibit largely restored osteoclastogenesis and bone mass, suggesting the activation of Trpc1 is required for normal osteoclast differentiation and the maintenance of bone density. These observations also indicate that Trpc1 channel in mice is inactivated in physiological conditions. Whether Trpc1 affects osteoclast development and functions in pathological conditions like tumors is required to be elucidated. Moreover, one might also need to consider the compensation effects among TRPCs due to their functional similarity when understanding a specific TRPC channel in modulation of osteoclastogenesis.

Taken together, TRPCs, especially TRPC1, play essential roles in modulation of tumor progression and osteoclast activation. Considering TRPC1 in osteoclasts is inactivated in physiological conditions and required for EMT in breast cancers, it will be interesting to examine whether TRPC1 is activated when bone metastasis is occurred in breast cancers. Moreover, such activation probably will lead to enhanced bone metastasis as TRPC1 promotes calcium influx that benefits both cancer cells metastasis and osteoclasts activation.

TRPVs

TRPVs in Breast Cancers

The mammalian TRPVs contain six members, named as TRPV1 to TRPV6. Unlike TRPCs, most TRPVs reported seem to act as the tumor suppressor in breast cancers. So far, TRPV1, TRPV4 and TPRV6 have been well-studied in breast cancers progression. It has been reported that TRPV1 activation via capsaicin together with MRS1477 largely reduces MCF-7 viability (41), which is not observed in primary breast epithelial cells, indicating TRPV1 is a potential drug target for treating breast cancers without affecting normal cells. Moreover, activation of TRPV1 also increases the anti-tumor efficiency of clinical drugs like doxorubicin probably via aggravating the ROS induced apoptosis (42). Importantly, TRPV1 is expressed in neurons and senses signals of pain (103), whereas tumor-induced bone pain is a severe clinical condition that needs to be addressed (104). Tong et al. show that TRPV1 is activated by formaldehyde secreted by the cancer tissues and induces metastatic bone cancer pain, especially in the condition of tumor acidic microenvironment (43, 44). These observations raise a complicated network among tumor cells, neurons, bones and the extracellular calcium pool in the metastatic bone microenvironment, which is required efforts to be further studied.

The role of TRPV4 in breast cancers is much complicated, making it difficult to be a potential drug target at present. Particularly, activation of TRPV4 induces both cell death and metastasis in breast cancer cells. One study reported that pharmacological activation of TRPV4 by GSK1016790A drastically enhances tumor cells death mainly via two routes: apoptosis mediated by PARP-1 cleavage and oncosis accompanied with a rapid decrease of intracellular ATP production (49). Interestingly, the expression of TRPV4 is correlated with poor clinical outcomes in breast cancers (50). Further studies show that the expression of TRPV4 in breast cancer cells lead to the actin reorganization and therefore promotes breast cancer cell softness and tumor invasion without affecting cell proliferation (50, 51). Similarly, TRPV4 is also

required for actin remodeling in endothelial cells and thus promotes angiogenesis and tumor progression (51). All in all, more efforts are required to precisely understand the molecular mechanism of TRPV4 in either enhancing tumor cell death or cytoskeleton reorganization, which might provide potential strategies for treating breast cancers by targeting TRPV4 in the future.

Unlike TRPV1 and TRPV4, TRPV6 has been shown to positively promote breast cancers progression. TRPV6 is widely upregulated in multiple malignant cells including breast cancer cells (57). Specifically, TRPV6 is highly expressed in estrogen receptor-negative breast tumors as well as HER2-positive tumors. Such expression is correlated with a low survival rate in breast cancer patients, which might be ascribed to the essential role of TRPV6 in driving abnormal tumor cell proliferation. Importantly, tamoxifen, the widely used drug in breast cancer therapy, is more sensitive in reducing cell viability when TRPV6 is silenced (58). Interestingly, tamoxifen also functions as a negative modulator of TRPV6 as it reduces the calcium influx mediated by TRPV6 (59). Therefore, TRPV6 could be a potential drug target that alleviate chemotherapeutic resistance in breast cancers. Though TRPV1, TRPV4, and TRPV6 all mediate calcium fluxes in breast cancer cells, their unique and even opposite functions in breast cancer progression suggest a complicated network of calcium signaling in modulation of tumor cell functions, which requires further efforts to elucidate. Understanding the specific mechanism of each TRPVs would benefit precise targeting strategies for treating breast cancers, especially in the condition with large amount of extracellular calcium pools, like the bone metastatic niche.

TRPVs in Osteoclasts

TRPVs have been well-studied in osteoclast differentiation and activation. Except TRPV3, TRPV1, TRPV2, TRPV4, TRPV5, and TRPV6 are all reported to modulate osteoclasts formation or functions. Multiple studies reported that TRPV1 is required for osteoclastogenesis and bone resorption. TRPV1 mediated calcium influx is accompanied with cannabinoid receptors (CB) activation in osteoclasts, including CB1 and CB2. Intriguingly, CB1 and TRPV1 facilitates osteoclast differentiation while CB2 inhibits osteoclast activation (45). Nevertheless, TRPV1 deficient mice exhibit largely reduced osteoclast numbers and increase bone mass *in vivo*. Further analysis reveal a reduction of intracellular calcium levels and calcium oscillations in osteoclast precursors stimulated with RANKL (46). As calcium oscillations and the following activated NFATc1 are the major signals for osteoclastogenesis (24), TRPV1 seems to act as one of the early-stage determinants for osteoclast differentiation. TRPV2 is another TRPV that benefits calcium oscillations during osteoclast differentiation. The expression of TRPV2 is gradually increased during osteoclastogenesis (47), suggesting that TRPV2 is an essential calcium channel to sustain the early-stage calcium signals in osteoclasts. Importantly, the expression of TRPV2 is even enhanced in bone marrow biopsy specimens from patients suffering multiple myeloma (MM) compared to healthy controls, which is correlated with a poor clinical outcome of MM patients accompanied with enhanced osteoclast activation (48). Unlike TRPV1 and TRPV2, TRPV4 and TRPV5 are reported as critical modulators of osteoclast differentiation and activation in the late stage. TRPV4 deficient mice also exhibit significantly reduced osteoclasts number and enhanced bone mass. Interestingly, though osteoclast differentiation is restricted in TRPV4 deficient cells, this defect is more prominent in large or late-stage differentiated osteoclasts (52), suggesting TRPV4 mainly functions at the late-stage of osteoclastogenesis. Further analysis indicates that together with calcium oscillations in the early stage, TRPV4 induced calcium influx sustain NFATc1 activation and therefore maintain osteoclast differentiation. The same author further identified a gain of function mutant (R616Q/V620I) of TRPV4 and found the mutated mice show opposite phenotype compared to TRPV4 deficient mice (53), further confirming the importance of TRPV4 in modulation of osteoclast activation. Intriguingly, nearly 70 TRPV4 mutants were identified and cause disease in human patients (54), from which most of them causing skeleton dysplasia are gain of function mutants, confirming the significance of TRPV4 in modulation of osteoclastogenesis in mice. Opposite to TRPV4, TRPV5 deficiency leads to an increase of osteoclast size and number, however, the bone resorption are totally blocked in TRPV5 deficient osteoclasts (55). As TRPV5 is mainly localized at the ruffled border membrane in osteoclasts (56), it is reasonable that TRPV5 is essential for the function of mature osteoclasts. The enlarged osteoclast size could be due to compensation for bone resorption, similar as cathepsin K deficiency (105). Interestingly, human cells knocking down TRPV5 after osteoclast maturation leads to an enhanced bone resorption, opposite to the phenotype observed in TRPV5 knockout osteoclasts in mice (56). This could be due to the different stage they silenced TRPV5 or species variations. Nevertheless, TRPV5 is an essential modulator of osteoclast activation in the late stage. TRPV5 and TRPV6 are highly homologous in TRPV subfamily, with 75% homology in amino acids. TRPV6 deficiency also leads to a large osteoclast size and an increase in osteoclast numbers. Unlike TRPV5, TRPV6 knockout osteoclasts show a largely increase bone resorption (60), suggesting TRPV6 is clearly a negative modulator of osteoclast differentiation and activation. TRPV5 and TRPV6 share high similarity and distinct from other TRPVs, however, exhibit different functions in osteoclasts. As TRPV6 does not whereas TRPV5 does affect calcium oscillations during osteoclast differentiation, the mechanism of these two channels in osteoclasts should exhibit their unique features that has not been described in other systems, which required further studies to clarify.

Taken together, TRPVs play essential roles in osteoclast differentiation and activation in a stage-dependent manner. Considering the expression of TRPVs is dynamically modulated during osteoclast differentiation or in pathological conditions, it will be interesting to first analyze the expression profile of TRPVs in osteoclasts as well as tumor cells during bone metastasis. This might give the evidence how these TRPVs differently modulate either tumor progression or osteoclast activation, two essential aspects for bone metastasis. Also, unlike TRPCs, most TRPVs reported show opposite functions between tumor cells and osteoclasts, which TRPVs restrict tumor progression and

are required for osteoclast activation. Interestingly, TRPV4 itself could both promote tumor cell death and metastasis, indicating that TRPVs might play totally different roles in certain scenario, and raising the possibility that certain TRPV, like TRPV4, would benefit both tumor cell invasion and osteoclastogenesis in the context of bone metastasis, which requires further efforts to elucidate.

TRPMs

TRPMs in Breast Cancers

The mammalian TRPM subfamily contains eight members, from TRPM1 to TRPM8. TRPMs have also been recognized as important modulators in multiple cancers progression (106), from which TRPM2, TRPM7, and TRPM8 have been shown to promote breast cancer development. TRPM2 has been well-recognized to promote cell death and tissue injury (107–109), however, improve the cell viability in breast cancer cell lines (61). Silencing TRPM2 in MCF-7 and MDA-MB-231 breast cancer cell lines induces significantly DNA damage compared to that in MCF-10A, the widely used non-cancerous breast cells. Interestingly, calcium influx is not significantly affected when TRPM2 is inhibited in breast cancer cells (61), indicating TRPM2 would not regulate bone metastasis in a calcium microenvironment dependent manner. TRPM7 is well-studied and modulates numerous functions in breast cancer progression, especially in cancer metastasis (62–64, 110). Two SNPs of TRPM7 have been shown to associate with breast cancer in Han population of northeast China (111), indicating the importance of TRPM7 in breast cancers progression. Indeed, Kaplan–Meier analysis in breast cancer patients found that the high expression of TRPM7 is significantly correlated with recurrence-free survival and distant metastasis-free survival in breast cancers (64). Further analysis show that silencing TRPM7 reduces breast cancer cells migration and metastasis by regulation of myosin II–based cellular tension and thus cell movement (112). Mechanistically, the kinase domain of TRPM7 is mainly responsible for modulation of breast cancer cells migration. Importantly, TRPM7 mediated calcium signals further modulate EMT in breast cancer cells, which TRPM7 deficiency specifically reduces EGF-induced STAT3 phosphorylation and the expression of Vimentin, suggesting that TRPM7 is required for maintaining a mesenchymal feature in breast cancer cells (110). Except TRPM7, TRPM8 has also been reported to modulate EMT in breast cancer cells. Overexpression of TRPM8 remarkably increases MCF-7 migration and the shape change in 3D sphere formation. Whereas, silencing TRPM8 largely reduces migration and the shape switch in MDA-MB-231 cells (66). Taken together, TRPMs most likely regulate breast cancer metastasis, though one study also indicate TRPM7 regulates breast cancer cell proliferation (65). And, it will be reasonable to further analyze how the modulation of metastasis by TRPMs eventually regulates bone metastasis.

TRPMs in Osteoclasts

Unfortunately, so far little has been shown of TRPMs in modulation of osteoclast differentiation and activation. Considering TRPMs could be regulated by environmental changes of ATP, PH, heat, lipids, and also associate with other calcium channels to modulate calcium homeostasis (96), it is highly possible that TRPMs play essential roles in osteoclast differentiation and activation. Understanding the functions and mechanism of TRPMs both in breast cancer cells and osteoclasts will be beneficial for clarifying the importance of calcium microenvironment acting as a vicious cycle for bone metastasis, which would provide new potential and efficient drug targets for treating breast cancers.

TRPA1, TRPPs, and TRPMLs

Compared to TRPC, TRPV, and TRPM subfamilies, little has been known about the other mammalian TRPs in modulation of breast cancers progression and osteoclasts activation, including TRPA, TRPP, and TRPML subfamilies. The mammalian TRPA subfamily only contains one member, named as TRPA1. The mammalian TRPP subfamily contains TRPP2, TRPP3, and TRPP5. And the mammalian TRPML subfamily contains TRPML1, TRPML2, and TRPML3 (96).

TRPA1 has been shown to be most highly upregulated among all of the TRPs in invasive ductal breast carcinoma, indicating TRPA1 promotes breast cancer progression. Indeed, TRPA1 accelerates breast cancer development in two routes. TRPA1 both enhances tumor growth and reduces chemo-sensitivity through mediating calcium influx dependent anti-apoptotic pathways (67). Similarly, TRPP2 has also been shown to promote drug resistance in breast cancers. Silencing TRPP2 does not affect the cell viability of breast cancer cells but impressively promotes the sensitivity of paclitaxel in treating MDA-MB-231 cells. TRPP2 is highly phosphorylated in breast cancer cells and treatment with paclitaxel further increases the phosphorylation level, which could be one of the mechanisms for the chemo-resistance (68). All in all, considering the drug resistance by TRPA1 and TRPP2 reported in breast cancers, these two channels probably are essential for tumor development in the late-stage, like bone metastasis. Their exact functions and mechanism in bone metastasis, especially in tumor induced osteoclastogenesis and osteolysis require further studies to elucidate.

TRPML1 is the only known cation channel in the TRPML subfamily (113). It has been shown that TRPML1 modulates calcium influx in lysosomes and is essential for osteoclastogenesis and bone resorption (69). Trpml1 deficient mice exhibit largely reduced osteoclasts and enhanced bone mass. *In vitro* analysis revealed that Trpml1 is required for lysosomal functions and therefore osteoclasts activation, probably via modulation of lysosomal calcium signals, one of the important sources for calcium oscillations and NFATc1 activation in osteoclasts. TRPML1 mediated lysosomal functions are also important in breast cancer development (70). TRPML1 is highly expressed in the triple negative breast cancer cells. Knocking down TRPML1 prevents lysosomal ATP exocytosis and therefore magnificently reduces tumor growth and migration. However, it is not clear whether TRPML1 promotes breast cancer progression in response to calcium signals. Considering the calcium mediated lysosomal functions and the related change of cellular metabolism mediated by organelle contacts are recently one of the most impressive fields in cancer development as well as

multiple physiological and pathological functions (114), it would be very interesting to understand the potential promotion of bone metastasis by TRPML1-mediated lysosomal calcium cascades in breast cancer cells and osteoclasts.

VOLTAGE-GATED CALCIUM CHANNELS (VGCCS)

The voltage-gated calcium channels are mostly permeable for calcium influx, with an extremely slight permeable for sodium ions in physiological conditions. VGCCs are mostly studied in excitable cells (115), like neurons and muscles, however, have also been shown to play essential roles in non-excitable cells (116), including breast cancers and osteoclasts. The activation of VGCCs requires membrane depolarization and mediates calcium influx to transduce downstream signals (117). VGCCs contain ten members, including $Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$, $Ca_v1.4$, $Ca_v2.1$, $Ca_v2.2$, $Ca_v2.3$, $Ca_v3.1$, $Ca_v3.2$, and $Ca_v3.3$. Depending on the cell types, VGCCs mediated calcium influx activates a variety of downstream targets for modulation of cellular functions.

The importance of VGCCs in modulation of breast cancers progression has been revealed by using an engineered VGCC lacking inactivation (Cec) (118), which triggers massive calcium influx and cell death in breast cancer cells but not in MCF-10A, the non-tumor human mammary epithelial cells. Importantly, the primary breast tumors generated by MDA-MB-231 are significantly shrank 2 weeks after infected with lentivirus containing Cec, indicating that activating VGCCs will be beneficial for the treatment of breast cancers. However, three VGCCs reported are all believed to promote cell proliferation or tumor growth in breast cancers (71, 73, 74, 119), including $Ca_v1.3$, $Ca_v3.2$, and $Ca_v3.3$. Whereas, $Ca_v3.1$ has been shown to act as a tumor suppressor gene in breast cancer cells by retarding proliferation and enhancing apoptosis (72), yet its exact role in tumor growth has not been investigated. Note the efficiency of VGCCs in promotion of breast cancers requires the constant activation of the channel, like the stimulation by extracellular pressure. For instance, cells only expressing Cec, the engineered $Ca_v1.2$ lacking inactivation, but not $Ca_v1.2$, largely induces cell death in breast cancers (118). Therefore, further studies are required to identify the stimuli for constant activation of VGCCs in breast cancers, or other tumor cells during bone metastasis, which is the important precondition for regulation of calcium homeostasis by VGCCs acting as a vicious cycle in bone metastasis.

STORE-OPERATED CALCIUM ENTRY (SOCE)

SOCE is an ubiquitous mechanism in non-excitable cells to modulate calcium homeostasis with important biological functions (120). During the last decade, the most important advance in SOCE field is the identification of ER-resident STIM1 (121–123) and plasma membrane (PM)-located ORAI1 (124–126) as two major components for SOCE activation. Their homology, STIM2, ORAI2, and ORAI3, have also been shown to participate in SOCE, yet the functions are minor or with controversy compared to STIM1 and ORAI1 (127). SOCE activation is a multistep process that requires the conformational change of STIM1 and ORAI1 (28). Particularly, STIM1 is inactivated in the ER by association with calcium via the EF-hand domain located in its N-terminal region. The decline or depletion of calcium in the ER due to either promotion of calcium release or reduction of calcium reuptake therefore leads to the conformational change and thus oligomerization of STIM1. The oligomerized STIM1 then redistributes to the ER-PM contact with the help of the cytoskeleton system, where it associates with the clustered ORAI1 and forms the channel for extracellular calcium entry, which ultimately refills the ER calcium storage to sustain calcium signals in the cells. SOCE therefore is an essential and specific process to maintain calcium homeostasis in cells when or after cells were activated by extracellular stimuli, which is essential for biological functions. Indeed, Stim1 or Orai1 deficiency in mice is embryonic lethal and numerous STIM1 or ORAI1 mutants have been identified in humans exhibiting disorders of calcium influx in cells. Loss- and gain-of function mutants both result in multiple severe disease in human patients (128), including immune disorders and skeleton abnormal development.

SOCE in Breast Cancers

Recent evidence further reveals the importance of SOCE in modulation of cancer progression, including breast cancers (75). SOCE is closely related to breast cancer metastasis, especially bone metastasis. Several studies found that SOCE facilitates migration and metastasis in breast cancers mainly via three routes. Yang et al. show that knocking down either STIM1 or ORAI1 in MDA-MB-231 decreases the invasion while overexpression of STIM1 and ORAI1 together in MCF-10A enhances it (76). This could be due to the impairment of assembly and disassembly of focal adhesions in STIM1 or ORAI1 deficient cells. Importantly, pharmacological inhibition of SOCE by SKF96365 significantly reduces breast cancer cell metastasis, giving an evidence that targeting SOCE could be a potential strategy for treating tumor metastasis. SOCE is also essential for the enolase-1 (ENO-1) exteriorization to the cell surface (77). The exteriorized ENO-1 modulates pericellular proteolysis and thus allows cells to invade tissues (129). Therefore, SOCE can also promote the migration and invasion of breast cancer cells via mediating the translocation of ENO-1 to cell surfaces. Finally, SOCE promotes TGFβ-induced EMT during breast cancer progression (78). Both STIM1 deficiency and pharmacological inhibition of SOCE by YM58433 reduce the expression of Vimentin but enhances the expression of E-cadherin stimulated with TGF-β in breast cancer cells, indicating that SOCE is required for maintaining the epithelial conditions in breast cancer cells and thus modulates tumor cell migration. Interestingly, one study found that SOCE is also slightly required for TGF-β induced cell cycle arrest in breast cancer cells (79). Dependent on the tumor stages, TGF-β signals have been shown to have opposite functions in breast cancer cells (130). Therefore, SOCE might also has similar effects that SOCE and TGF-β signals synergistically restrict breast cancer cell proliferation in

the early stage, whereas in the late stage, SOCE modulates TGF-β induced EMT and tumor metastasis. The importance of SOCE in modulation of breast cancers progression has also been revealed by analyzing the clinical relevance in human samples. Both STIM1 and ORA11 express highly in breast cancer cells and their high expression are correlated with tumor aggressiveness and poor prognosis of breast cancers (131). In addition, three studies reported multiple SNPs of ORAI1 in breast cancers, which were predicted to associate with tumor malignancy (132–134). Overall, SOCE activation has been shown to promote breast cancer progression, especially via enhancing the tumor metastasis.

SOCE in Osteoclasts

SOCE has also been shown to be a critical signal for calcium oscillations during osteoclast differentiation. Knocking down Stim1 in pre-osteoclasts dramatically reduces calcium oscillations (80), the essential signals for osteoclast differentiation. Orai1 deficiency also shows reduction of SOCE, impairment of NFATc1 translocation and defect of pre-osteoclasts fusion as well as osteoclastogenesis (81–83).

Combined with the observation of SOCE in modulation of cancer metastasis and osteoclastogenesis, it will be reasonable to speculate that SOCE activation would lead to bone metastasis. Indeed, one study show that the SK3, a potassium channel, associates with ORAI1 in lipid rafts and controls the constitutive calcium entry and thus bone metastasis in breast cancers (84). Another study found that SGK1 is essential for ORAI1 expression and therefore modulates calcium entry and osteoclastogenesis, which ultimately benefits bone metastasis (135). Therefore, SOCE could be essential signals for bone metastasis and targeting SOCE would be a potential strategy to treat this disease. However, since SOCE activation is widely required in non-excitable cells, targeting SOCE itself would lead to severe side effects. The good news is that multiple modulators have been identified to either promote or restrict SOCE activation (136–144), which would be potential targets for treating breast cancers without totally blocking SOCE activation. Further studies are required to clarify the exact roles and mechanism of these SOCE modulators in breast cancers progression, especially the bone metastasis.

P2X Receptors

P2X receptors are ligand-gated ion channels that are principally activated by ATP. So far seven members of this subfamily have been identified, which are numbered P2X1 through P2X7. Activation of P2X receptors by ATP would lead to trimerization of these receptors for cations entry, such as sodium or calcium ions. Both homo-trimers and hetero-trimers of these receptors have been reported (145). Similar as VGCCs, P2X receptors modulate calcium entry mostly in excitable cells, however, have also been shown to participate in regulation of tumor progression and osteoclastogenesis, especially the P2X7 receptor.

P2X7 in Breast Cancers

The P2X7 receptor modulates proliferation, apoptosis, migration, invasion and metastasis in breast cancers. The P2X7 receptor could be activated by ATP that is rich in the tumor microenvironment, leading to the downregulation of E-cadherin and upregulation of MMP-13 mediated by the PI3K-AKT cascade in T47D breast cancer cells (85). This is one of the mechanisms shown to promote breast cancer cell invasion by the P2X7 receptor. Activation of the P2X7 receptor also changes the morphology of MDA-MB-435 cells, which prolongs the cell shape facilitating cell migration. Interestingly, P2X7 mediated cell migration but not cell extension is largely reduced in SK3 deficient cells. Nevertheless, P2X7 enhanced cell invasion could also be mediated by the SK3 channel (86). A recent study further suggested that P2X7 promotes cell migration and metastasis via increasing the extracellular vesicles production in tamoxifen-resistant breast cancers (87), further indicating the possibility to target P2X7 as a strategy for alleviating drug resistance in breast cancers. Except cell invasion, the P2X7 receptor also increases cell proliferation and reduces cell apoptosis in breast cancer cells. Knocking down P2X7 in MCF-7 cells almost blocks cell viability and significantly increases apoptosis (88). One study further found that P2X7 exists a distinct conformational form that restricts the large pore opening in tumor cells, named as non-pore functional P2X7 (nfP2X7). The nfP2X7 signal is essential for breast cancer cell survival and proliferation but has limited calcium entry therefore declines cell death (89). It is interesting to observe that nfP2X7 in the breast cancer cells has similar functions as P2X7 except calcium entry, which indicates that modulation of the calcium microenvironment would directly affects breast cancer cell viability as we proposed initially. It would be more interesting to analyze whether cancer cells have these type of non-functional P2Xs in cancer cells migrated to the osteolytic tissues where amounts of calcium ions exist, sustaining the benefits from calcium signaling for tumor growth but limiting the excessive calcium entry that leads to cancer cell death.

P2X7 in Osteoclasts

In addition to breast cancer cells, the P2X7 receptor is also well-studied in modulation of osteoclastogenesis. The P2X7 receptor links the extracellular stimulus and osteoclast activation, which the mechanical and other stimuli leads to nucleotides release, including ATP, and activates osteoclastogenesis via ATP mediated activation of P2X7 and the downstream NF-κB cascade (90). Interestingly, osteoclasts are normally fused and differentiated in P2X7 knockout mice (91), indicating that P2X7 is maintained inactive in physiological conditions but largely activated in pathological conditions. Indeed, it has been reported that compared to WT mice, the P2X7 deficient mice exhibit largely reduced bone mass and increased osteoclast numbers in OVX-mediated osteoporosis model, but not in the SHAM control (92). Another study further revealed that P2X7 drives pre-osteoclast fusion in response to amount of ATP stimulation (93), indicating that P2X7 might be a critical signal for pathological osteoclast activation and bone remodeling as damaged bone would release a large number of ATP and other nucleotides. In humans, several SNPs of P2X7 have been identified to associate with osteoporosis in postmenopausal woman (146, 147) as well as the risk of fracture (147, 148). Pharmacological inhibition of P2X7 significantly inhibits human osteoclasts formation (94), suggesting the importance of P2X7 activation in modulation of bone disease in human.

Taken together, P2X7 plays important roles in osteoclast activation specifically in pathological conditions, raising the possibility that P2X7 would play essential roles in bone metastasis. Actually Zhou et al. have shown adenosine nucleotides promotes breast cancer growth and bone metastasis in a high dosage (95). Interestingly, this study found that ATP mediated activation of P2X7 inhibits MDA-MB-231 cells migration, raising a possibility that P2X7 might be essential for tumor cell residence in osteolytic niche after bone metastasis, which requires further studies to investigate.

EXTRACELLULAR CALCIUM ENTRY IN OSTEOBLASTS

Calcium homeostasis are also important signals in modulation of osteoblast differentiation and functions. Till now, though not as much as those reported in breast cancer cells and osteoclasts, multiple calcium channels have also been reported to modulate osteoblast proliferation, differentiation, migration and mineralization, including TRPV1, TRPV4, TRPM7, TRPP2, Cav1.2, ORAI1/SOCE, P2X1, and P2X7. He et al., reported that TRPV1 deficient BMSCs exhibited impaired osteoblast differentiation and mineralization *in vitro*. As a result, TRPV1 deficiency leads to delayed fracture healing in the pathological mice model (46). TRPV4 has also been indicated in regulation of osteoblast activity, which TRPV4 is highly induced in differentiated osteoblasts and essential for calcium oscillations in osteoblasts (149). Though the importance of TRPV4-induced calcium oscillations in regulation of osteoblast activity have not been well-clarified yet, considering calcium oscillations are one of the important features in mature osteoblast and osteocytes in response to mechanical force (150), it would be reasonable to speculate that TRPV4 is essential for mature osteoblast activity, which requires further efforts to elucidated. Similar as TRPV4, TRPM7 is also upregulated during osteoblast differentiation. TRPM7 deficiency has been reported to result in defects in osteoblast proliferation, differentiation and mineralization, however, such functions might be via not only calcium but also magnesium entry (151, 152). TRPP2 is another TRP that has been reported to be essential for osteoblast differentiation and mineralization. The TRPP2 conditional knockout mice exhibit significantly reduced bone mass both in trabecular and cortical bone (153). These observations raise the importance of TRPs in modulation of osteoblast activity as all of the TRPs reported are require for osteoblast differentiation and mineralization. In addition to TRPs, Cav1.2 (154), ORAI1 (155, 156), and P2X1 (157) have also been shown to promote osteoblast differentiation and mineralization *in vitro* and *in vivo*, suggesting extracellular calcium entry is require for the maintenance of osteoblast activity.

While abnormal osteoblastogenesis is more frequently observed in prostate cancers driven bone metastasis, the major function of osteoblasts during bone metastasis in breast cancers has been believed to enhance RANKL expression but reduce OPG expression stimulated by tumor cells, and therefore facilitate osteoclastogenesis and tumor cells metastasis (7). Though extracellular calcium entry is required for osteoblast differentiation and maturation, it would be more important to explore whether calcium channels modulate RANKL/OPG expression in osteoblasts. Interestingly, high dietary calcium administration in mice leads to enhanced osteoblastic bone formation and slightly but significantly reduces RANKL/OPG ratio in bone extracts (158). Another study further finds that TRPV1 activation promotes OPG expression but not affects RANKL expression, which leads to a reduced RANKL/OPG ratio (159), similar as the dietary calcium administration. Also, the gain of function mutated Cav1.2 mice (Prx1-Cre driven) exhibit increased serum OPG concentrations and the isolated BMSCs show reduced RANKL/OPG expression. Importantly, the Cav1.2 mutated calvaria osteoblasts exhibit defects in promotion of osteoclastogenesis in the co-culture system *in vitro* (154). Taken together, it seems that extracellular calcium entry in osteoblasts would suppress RANKL/OPG ratio, osteoclastogenesis and therefore benefit bone formation. It would be reasonable to assume that extracellular calcium entry would act as a negative factor in the scenario of osteoblasts-mediated bone metastatic niche formation in breast cancers. However, as the reduced RANKL/OPG ratio reported is slightly modulated by extracellular calcium, further studies are required to clarify whether the extracellular calcium modulated RANKL/OPG ratio produced in osteoblasts would be compensated by other major signals like cytokines secreted from breast cancer cells during bone metastasis, and therefore plays minor effects in the whole vicious cycle.

CALCIUM CHANNELS AND BONE METASTASIS IN THE SPECIFIC SUBTYPE OF BREAST CANCERS

One of the largest issues in tumors is the heterogeneity that tumors are a mixture of different type of cells with different molecular makers (160). Thanks to the efforts made by numerous researchers, several classifications have been developed to categorize breast cancers (161, 162), including the immunohistochemical subtype characterized by the expression of estrogen receptors (ER), progesterone (PR) and epidermal growth factor receptor 2 (HER2). According to the expression of these three receptors, the breast cancers can be categorized as Luminal A (ER+PR+HER2-), Luminal B (ER+PR+HER2+), HER2+ (ER-PR-HER2+), and triple negative breast cancers (TNBC, ER-PR-HER2-). These four subtypes of breast cancers also exhibit different potency of bone metastasis (160). Overall, the luminal and Her2+ subtype of breast cancers exhibit high potency of bone metastasis compared to the basal-like tumors, the major components (around 75%) in TNBC subtype. Importantly, no luminal subtype has been observed in TNBC subtype. In another word, ER/PR+ and Her2+ tumors show high potency of bone metastasis compared to the TNBC subtype.

Interestingly, the expression of calcium channels is dynamically regulated in these three subtypes and have been well-summarized recently (163, 164). Particularly, TRPC1, STIM1, and ORAI1 are downregulated in luminal and Her2+

subtypes but upregulated in the TNBC subtype. Whereas, the expression of Cav3.2 is upregulated in luminal and Her2+ subtypes but downregulated in the TNBC subtype. These observations indicate that VGCCs might be essential signals in modulation of calcium homeostasis and therefore bone metastasis in luminal and Her2+ subtypes. In addition, one cannot exclude the possibility that the low expression/activation of TRPs and SOCE in luminal and Her2+ subtypes is the result of a negative feedback, which the over-activation of TRPs and SOCE strongly enhance calcium influx in breast cancers and thus bone metastasis, however, too much calcium burden in turn declines the expression of calcium channels (TRPs, STIM1, and ORA1) to balance the intracellular calcium homeostasis, which has been reported in other cation channels (165). In conclusion, further studies are required to utilize *in vivo* mice model or clinical samples but not breast cancer cell lines alone to elucidate the importance of VGCCs/TRPs/SOCE in luminal and Her2+ subtypes in the context of bone metastasis.

All in all, all of the four calcium channels have shown their potency in modulation of breast cancers progression, including tumor cells viability and migration, osteoclasts activation, and bone metastasis. Further studies are required to elucidate that (1) how does these channels respond to the calcium microenvironment and tumor progression during bone metastasis? (2) Do these channels synergistically or independently modulate bone metastasis? (3) Do these channels modulate bone metastasis in a time- and space- dependent manner? (4) Do these channels dynamically modulated in specific subtypes of breast cancers and related bone metastasis? It is worth noting that since tumor cells and osteoclasts are non-excitable cells, SOCE would be an extremely important modulator for bone metastasis as SOCE mainly regulates calcium homeostasis in non-excitable cells. Moreover, SOCE normally modulates biological functions synergistically with cascades declining or depleting the ER calcium storage, therefore modulation of SOCE activity would not heavily hurt physiological activities of cytosolic calcium signals in a short range, leading to limited side effects in treating bone metastasis.

TREATING BONE METASTASIS BY CONSIDERING SYSTEMIC CALCIUM HOMEOSTASIS

The calcium channels have already been shown to be critical modulators for breast cancers progression and osteoclasts activation. Multiple reports have shown the potency of agonists or antagonists targeting calcium transporters in treating cancers both in mice models and in pre-clinical studies (15, 166). Also, studies summarized above mentioned that several agonists or antagonists targeting these channels would affect breast cancers progression in *vitro* and *in vivo*, raising the possibility that these compounds could be potential drugs for treating breast cancers and even bone metastasis. Here we will not overview again these agonists or antagonists, but focus more on the known drugs treating bone metastasis in sight of modulation of systemic calcium homeostasis, including bisphosphonates, and denosumab.

Bisphosphonates are a family of drugs that suppress osteoclasts-mediated bone resorption and alleviate abnormal bone loss in multiple diseases (167). Now it has been well-recognized that bisphosphonates inhibit bone resorption mainly via four routes, including preventing the recruitment, inhibiting the adhesion, shortening the lifespan and reducing the activity of osteoclasts (168). Bisphosphonates were discovered for mainly treating osteoporosis but have also been shown to be effective in osteoclasts-related disease, including bone metastasis in breast cancers. Bisphosphonates exhibit dual functions in bone metastasis. On the one hand, it alleviates bone loss and relevant bone pain therefore suppresses serum calcium concentrations. On the other hand, bisphosphonates have been reported to induce apoptosis and suppress invasion in tumor cells therefore ameliorate tumor growth (169). Importantly, treatment of bisphosphonates in patients not only alleviates bone loss but also results in hypocalcemia, a status due to reduced calcium loss from bones (167). So far the reduction of serum calcium concentrations due to bisphosphonates treatment have been well-recognized. The secondary effects of this reduction that the decline of extracellular calcium microenvironment would counteract on the activity of tumor cells and osteoclasts require further studies to elucidate. This is important as hypocalcemia is a short-lasting event and serum calcium concentrations would be recovered due to a negative feedback that parathyroid hormone is increased for upregulation of calcium absorption (170). Considering the serum calcium concentration would play crucial roles in bone metastasis via the calcium channels, the duration for administration of calcium or Vitamin D to treat hypocalcemia due to bisphosphonates treatment might need to be reconsidered in patients suffering bone metastasis.

Denosumab is another drug treating bone loss by targeting osteoclasts-mediated bone resorption. Denosumab is an antibody targeting human but not rodent RANKL, the well-known essential ligands for osteoclastogenesis, and therefore is utilized to treat multiple osteoclasts-related disease, including bone metastasis (171, 172). Indeed, the scope of application of denosumab in clinic trial includes osteoporosis and multiple types of cancers developing bone metastasis. Similar as bisphosphonates, administration of denosumab efficiently alleviates bone loss and reduces bone metastasis. In a pre-specified interim analysis of giant cell tumors, 99% patients have been shown to respond to exhibit no disease progression after 12-months treatment (173), suggesting the high efficiency of denosumab in treating osteoclasts-related tumor progression. Interestingly, administration of denosumab also leads to hypocalcemia, but with a pretty low incidence in osteoporosis while a relatively high percentage in cancers (172). This might be due to the extremely high activation of osteoclasts and hypercalcemia in cancer patients. Further studies are required to clarify if tumors are more coordinated to hypercalcemia that leads to its malignancy including bone metastasis, and treatment of denosumab could nicely and specifically block the vicious cycle raised by the abnormal calcium homeostasis in bone metastasis.

Other inhibitors targeting bone resorption or RANKL-RANK signaling have also been reported to be effective in treating tumor progression and bone metastasis, including OPG-Fc (172), RANK-Fc (172), calcitonin (174), etc. Based on the observation of bisphosphonates and denosumab in treating bone metastasis, it will be reasonable to speculate that all of the other inhibitors modulation of hypercalcemia in cancer patients would likely lead to hypocalcemia. Therefore, it would be interesting to clarify if these inhibitors also alleviate tumor progression by affecting the calcium microenvironment during bone metastasis, which might provide efficient strategies of combinational therapies to synergistically treat cancers.

CONCLUSION AND PERSPECTIVE

Hypercalcemia has been recognized as the results of bone resorption during tumor progression. Raising of serum calcium concentrations in patients suffering hypercalcemia would lead to multiple disorders, which severely affects human health and even leads to mortality (19, 175). The importance of hypercalcemia in direct modulation of tumor progression has not been well-evaluated yet. Considering that the extracellular calcium and the related calcium channels have multiple functions in regulation of tumor progression and osteoclastogenesis, it would be reasonable to hypothesize that hypercalcemia in cancer patients further aggravates the tumor progression via the abnormal calcium homeostasis forming a vicious cycle among tumor cells and osteoclasts during bone metastasis, which could be one of the reasons to explain the high malignancy of tumor progression in patients suffering hypercalcemia. TRPs, VGCCs, SOCE and P2Xs are four major channels for calcium entry and play important roles in tumor cells proliferation, survival, migration and metastasis. Also, these four channels modulate osteoclast differentiation and activation in certain scenarios. Though different functions of these four channels have been observed in modulation of the activity in cancer cells and osteoclasts, most of them show their capacity in promoting tumor progression and osteoclast activation. Therefore, further efforts are required to elucidate the exact functions and mechanism of these four channels in bone metastasis, especially SOCE due to its specificity for calcium entry and its omnipresence in non-excitable cells. Understanding the vicious cycle of calcium homeostasis in bone metastasis mediated by these calcium channels would further provide potential combinational strategies together with the known chemotherapeutic treatments to treat cancers, including targeting the functional calcium channels or modulating the serum calcium concentrations.

AUTHOR CONTRIBUTIONS

ZYa and ZYu generated the concept and wrote the manuscript. XM and ZX searched the literatures and reviewed the manuscript. All of the authors approved the final manuscript.

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REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA*. (2018) 68:394–424. doi: 10.3322/caac.21492
2. Mehlen P, Puisieux A. Metastasis: a question of life or death. *Nat Rev Cancer*. (2006) 6:449–58. doi: 10.1038/nrc1886
3. Perez-Garcia J, Munoz-Couselo E, Cortes J. Bone metastases: causes, consequences and therapeutic opportunities. *EJC Suppl*. (2013) 11:254–6. doi: 10.1016/j.ejcsup.2013.07.035
4. Weilbaecher KN, Guise TA, McCauley LK. Cancer to bone: a fatal attraction. *Nat Rev Cancer*. (2011) 11:411–25. doi: 10.1038/nrc3055
5. Lambert AW, Pattabiraman DR, Weinberg RA. Emerging biological principles of metastasis. *Cell*. (2017) 168:670–91. doi: 10.1016/j.cell.2016.11.037
6. Lyle LT, Lockman PR, Adkins CE, Mohammad AS, Sechrest E, Hua E, et al. Alterations in pericyte subpopulations are associated with elevated blood-tumor barrier permeability in experimental brain metastasis of breast cancer. *Clin Cancer Res*. (2016) 22:5287–99. doi: 10.1158/1078-0432.CCR-15-1836
7. Suva LJ, Washam C, Nicholas RW, Griffin RJ. Bone metastasis: mechanisms and therapeutic opportunities. *Nat Rev Endocrinol*. (2011) 7:208–18. doi: 10.1038/nrendo.2010.227
8. Fox SB, Leek RD, Bliss J, Mansi JL, Gusterson B, Gatter KC, et al. Association of tumor angiogenesis with bone marrow micrometastases in breast cancer patients. *J Natl Cancer Instit*. (1997) 89:1044–9. doi: 10.1093/jnci/89.14.1044
9. Silva R, D'Amico G, Hodivala-Dilke KM, Reynolds LE. Integrins: the keys to unlocking angiogenesis. *Arteriosc Thromb Vasc Biol*. (2008) 28:1703–13. doi: 10.1161/ATVBAHA.108.172015
10. Catena R, Luis-Ravelo D, Anton I, Zandueta C, Salazar-Colocho P, Larzabal L, et al. PDGFR signaling blockade in marrow stroma impairs lung cancer bone metastasis. *Cancer Res*. (2011) 71:164–74. doi: 10.1158/0008-5472.CAN-10-1708
11. Sarvaiya PJ, Guo D, Ulasov I, Gabikian P, Lesniak MS. Chemokines in tumor progression and metastasis. *Oncotarget*. (2013) 4:2171–85. doi: 10.18632/oncotarget.1426
12. Sosnoski DM, Krishnan V, Kraemer WJ, Dunn-Lewis C, Mastro AM. Changes in cytokines of the bone microenvironment during breast cancer metastasis. *Int J Breast Cancer*. (2012) 2012:160265. doi: 10.1155/2012/160265
13. Coniglio SJ. Role of tumor-derived chemokines in osteolytic bone metastasis. *Front Endocrinol*. (2018) 9:313. doi: 10.3389/fendo.2018.00313
14. Monteith GR, Davis FM, Roberts-Thomson SJ. Calcium channels and pumps in cancer: changes and consequences. *J Biol Chem*. (2012) 287:31666–73. doi: 10.1074/jbc.R112.343061
15. Monteith GR, McAndrew D, Faddy HM, Roberts-Thomson SJ. Calcium and cancer: targeting Ca^{2+} transport. *Nat Rev Cancer*. (2007) 7:519–30. doi: 10.1038/nrc2171

16. Berridge MJ, Bootman MD, Roderick HL. Calcium signalling: dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol.* (2003) 4:517–29. doi: 10.1038/nrm1155
17. Doherty AH, Ghalambor CK, Donahue SW. Evolutionary physiology of bone: bone metabolism in changing environments. *Physiology.* (2015) 30:17–29. doi: 10.1152/physiol.00022.2014
18. Veldurthy V, Wei R, Oz L, Dhawan P, Jeon YH, Christakos S. Vitamin D, calcium homeostasis and aging. *Bone Res.* (2016) 4:16041. doi: 10.1038/boneres.2016.41
19. Stewart AF. Clinical practice. Hypercalcemia associated with cancer. *N Engl J Med.* (2005) 352:373–9. doi: 10.1056/NEJMcp042806
20. Cheng H, Lederer WJ. Calcium sparks. *Physiol Rev.* (2008) 88:1491–545. doi: 10.1152/physrev.00030.2007
21. Sims CE, Allbritton NL. Metabolism of inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate by the oocytes of Xenopus laevis. *J Biol Chem.* (1998) 273:4052–8. doi: 10.1074/jbc.273.7.4052
22. Feske S. Calcium signalling in lymphocyte activation and disease. *Nat Rev Immunol.* (2007) 7:690–702. doi: 10.1038/nri2152
23. Clapham DE. Calcium signaling. *Cell.* (2007) 131:1047–58. doi: 10.1016/j.cell.2007.11.028
24. Negishi-Koga T, Takayanagi H. Ca2+-NFATc1 signaling is an essential axis of osteoclast differentiation. *Immunol Rev.* (2009) 231:241–56. doi: 10.1111/j.1600-065X.2009.00821.x
25. Lewis RS. Calcium oscillations in T-cells: mechanisms and consequences for gene expression. *Biochem Soc Trans.* (2003) 31(Pt 5):925–9. doi: 10.1042/bst0310925
26. Mao D, Epple H, Uthgenannt B, Novack DV, Faccio R. PLCgamma2 regulates osteoclastogenesis via its interaction with ITAM proteins and GAB2. *J Clin Invest.* (2006) 116:2869–79. doi: 10.1172/JCI28775
27. Yang Z, Kim S, Mahajan S, Zamani A, Faccio R. Phospholipase cgamma1 (PLCgamma1) controls osteoclast numbers via colony-stimulating factor 1 (CSF-1)-dependent diacylglycerol/beta-catenin/CyclinD1 pathway. *J Biol Chem.* (2017) 292:1178–86. doi: 10.1074/jbc.M116.764928
28. Soboloff J, Rothberg BS, Madesh M, Gill DL. STIM proteins: dynamic calcium signal transducers. *Nat Rev Mol Cell Biol.* (2012) 13:549–65. doi: 10.1038/nrm3414
29. Park CY, Hoover PJ, Mullins FM, Bachhawat P, Covington ED, Raunser S, et al. STIM1 clusters and activates CRAC channels via direct binding of a cytosolic domain to Orai1. *Cell.* (2009) 136:876–90. doi: 10.1016/j.cell.2009.02.014
30. Stathopulos PB, Schindl R, Fahrner M, Zheng L, Gasmi-Seabrook GM, Muik M, et al. STIM1/Orai1 coiled-coil interplay in the regulation of store-operated calcium entry. *Nat Commun.* (2013) 4:2963. doi: 10.1038/ncomms3963
31. Ma G, Wei M, He L, Liu C, Wu B, Zhang SL, et al. Inside-out Ca(2+) signalling prompted by STIM1 conformational switch. *Nat Commun.* (2015) 6:7826. doi: 10.1038/ncomms8826
32. Azimi I, Milevskiy MJG, Kaemmerer E, Turner D, Yapa K, Brown MA, et al. TRPC1 is a differential regulator of hypoxia-mediated events and Akt signalling in PTEN-deficient breast cancer cells. *J Cell Sci.* (2017) 130:2292–305. doi: 10.1242/jcs.196659
33. Schaar A, Sukumaran P, Sun Y, Dhasarathy A, Singh BB. TRPC1-STIM1 activation modulates transforming growth factor beta-induced epithelial-to-mesenchymal transition. *Oncotarget.* (2016) 7:80554–67. doi: 10.18632/oncotarget.12895
34. El Hiani Y, Ahidouch A, Lehen'kyi V, Hague F, Gouilleux F, Mentaverri R, et al. Extracellular signal-regulated kinases 1 and 2 and TRPC1 channels are required for calcium-sensing receptor-stimulated MCF-7 breast cancer cell proliferation. *Cell Physiol Biochem.* (2009) 23:335–46. doi: 10.1159/000218179
35. Davis FM, Peters AA, Grice DM, Cabot PJ, Parat MO, Roberts-Thomson SJ, et al. Non-stimulated, agonist-stimulated and store-operated Ca2+ influx in MDA-MB-468 breast cancer cells and the effect of EGF-induced EMT on calcium entry. *PLoS ONE.* (2012) 7:e36923. doi: 10.1371/journal.pone.0036923
36. Ong EC, Nesin V, Long CL, Bai CX, Guz JL, Ivanov IP, et al. A TRPC1 protein-dependent pathway regulates osteoclast formation and function. *J Biol Chem.* (2013) 288:22219–32. doi: 10.1074/jbc.M113.459826
37. Ma X, Chen Z, Hua D, He D, Wang L, Zhang P, et al. Essential role for TrpC5-containing extracellular vesicles in breast cancer with chemotherapeutic resistance. *Proc Natl Acad Sci USA.* (2014) 111:6389–94. doi: 10.1073/pnas.1400272111
38. Wang T, Ning K, Lu TX, Sun X, Jin L, Qi X, et al. Increasing circulating exosomes-carrying TRPC5 predicts chemoresistance in metastatic breast cancer patients. *Cancer Sci.* (2017) 108:448–54. doi: 10.1111/cas.13150
39. Zhang P, Liu X, Li H, Chen Z, Yao X, Jin J, et al. TRPC5-induced autophagy promotes drug resistance in breast carcinoma via CaMKKbeta/AMPKalpha/mTOR pathway. *Scient Rep.* (2017) 7:3158. doi: 10.1038/s41598-017-03230-w
40. Jardin I, Diez-Bello R, Lopez JJ, Redondo PC, Salido GM, Smani T, et al. TRPC6 channels are required for proliferation, migration and invasion of breast cancer cell lines by modulation of orai1 and orai3 surface exposure. *Cancers.* (2018) 10:E331. doi: 10.3390/cancers10090331
41. Naziroglu M, Cig B, Blum W, Vizler C, Buhala A, Marton A, et al. Targeting breast cancer cells by MRS1477, a positive allosteric modulator of TRPV1 channels. *PLoS ONE.* (2017) 12:e0179950. doi: 10.1371/journal.pone.0179950
42. Kosar PA, Naziroglu M, Ovey IS, Cig B. Synergic effects of doxorubicin and melatonin on apoptosis and mitochondrial oxidative stress in MCF-7 breast cancer cells: involvement of TRPV1 channels. *J Membr Biol.* (2016) 249:129–40. doi: 10.1007/s00232-015-9855-0
43. Pecze L, Josvay K, Blum W, Petrovics G, Vizler C, Olah Z, et al. Activation of endogenous TRPV1 fails to induce overstimulation-based cytotoxicity in breast and prostate cancer cells but not in pain-sensing neurons. *Biochim Biophys Acta.* (2016) 1863:2054–64. doi: 10.1016/j.bbamcr.2016.05.007
44. Tong Z, Luo W, Wang Y, Yang F, Han Y, Li H, et al. Tumor tissue-derived formaldehyde and acidic microenvironment synergistically induce bone cancer pain. *PLoS ONE.* (2010) 5:e10234. doi: 10.1371/journal.pone.0010234
45. Rossi F, Tortora C, Punzo F, Bellini G, Argenziano M, Di Paola A, et al. The endocannabinoid/endovanilloid system in bone: from osteoporosis to osteosarcoma. *Int J Mol Sci.* (2019) 20:1919. doi: 10.3390/ijms20081919
46. He LH, Liu M, He Y, Xiao E, Zhao L, Zhang T, et al. TRPV1 deletion impaired fracture healing and inhibited osteoclast and osteoblast differentiation. *Scient Rep.* (2017) 7:42385. doi: 10.1038/srep42385
47. Kajiya H, Okamoto F, Nemoto T, Kimachi K, Toh-Goto K, Nakayana S, et al. RANKL-induced TRPV2 expression regulates osteoclastogenesis via calcium oscillations. *Cell Calcium.* (2010) 48:260–9. doi: 10.1016/j.ceca.2010.09.010
48. Bai H, Zhu H, Yan Q, Shen X, Lu X, Wang J, et al. TRPV2-induced Ca(2+)-calcineurin-NFAT signaling regulates differentiation of osteoclast in multiple myeloma. *Cell Commun Signal.* (2018) 16:68. doi: 10.1186/s12964-018-0280-8
49. Peters AA, Jamaludin SYN, Yapa K, Chalmers S, Wiegmans AP, Lim HF, et al. Oncosis and apoptosis induction by activation of an overexpressed ion channel in breast cancer cells. *Oncogene.* (2017) 36:6490–500. doi: 10.1038/onc.2017.234
50. Lee WH, Choong LY, Mon NN, Lu S, Lin Q, Pang B, et al. TRPV4 regulates breast cancer cell extravasation, stiffness and actin cortex. *Scient Rep.* (2016) 6:27903. doi: 10.1038/srep27903
51. Fiorio Pla A, Ong HL, Cheng KT, Brossa A, Bussolati B, Lockwich T, et al. TRPV4 mediates tumor-derived endothelial cell migration via arachidonic acid-activated actin remodeling. *Oncogene.* (2012) 31:200–12. doi: 10.1038/onc.2011.231
52. Masuyama R, Vriens J, Voets T, Karashima Y, Owsianik G, Vennekens R, et al. TRPV4-mediated calcium influx regulates terminal differentiation of osteoclasts. *Cell Metab.* (2008) 8:257–65. doi: 10.1016/j.cmet.2008.08.002
53. Masuyama R, Mizuno A, Komori H, Kajiya H, Uekawa A, Kitaura H, et al. Calcium/calmodulin-signaling supports TRPV4 activation in osteoclasts and regulates bone mass. *J Bone Mineral Res.* (2012) 27:1708–21. doi: 10.1002/jbmr.1629
54. McNulty AL, Leddy HA, Liedtke W, Guilak F. TRPV4 as a therapeutic target for joint diseases. *Naunyn-Schmiedeberg's Arch Pharmacol.* (2015) 388:437–50. doi: 10.1007/s00210-014-1078-x
55. van der Eerden BC, Hoenderop JG, de Vries TJ, Schoenmaker T, Buurman CJ, Uitterlinden AG, et al. The epithelial Ca2+ channel TRPV5 is essential for proper osteoclastic bone resorption. *Proc Natl Acad Sci USA.* (2005) 102:17507–12. doi: 10.1073/pnas.0505789102

56. Chamoux E, Bisson M, Payet MD, Roux S. TRPV-5 mediates a receptor activator of NF-kappaB (RANK) ligand-induced increase in cytosolic Ca2+ in human osteoclasts and down-regulates bone resorption. *J Biol Chem.* (2010) 285:25354–62. doi: 10.1074/jbc.M109.075234
57. Peters AA, Simpson PT, Bassett JJ, Lee JM, Da Silva L, Reid LE, et al. Calcium channel TRPV6 as a potential therapeutic target in estrogen receptor-negative breast cancer. *Mol Cancer Therap.* (2012) 11:2158–68. doi: 10.1158/1535-7163.MCT-11-0965
58. Bolanz KA, Hediger MA, Landowski CP. The role of TRPV6 in breast carcinogenesis. *Mol Cancer Therap.* (2008) 7:271–9. doi: 10.1158/1535-7163.MCT-07-0478
59. Bolanz KA, Kovacs GG, Landowski CP, Hediger MA. Tamoxifen inhibits TRPV6 activity via estrogen receptor-independent pathways in TRPV6-expressing MCF-7 breast cancer cells. *Mol Cancer Res.* (2009) 7:2000–10. doi: 10.1158/1541-7786.MCR-09-0188
60. Chen F, Ni B, Yang YO, Ye T, Chen A. Knockout of TRPV6 causes osteopenia in mice by increasing osteoclastic differentiation and activity. *Cell Phy Biochem.* (2014) 33:796–809. doi: 10.1159/000358653
61. Hopkins MM, Feng X, Liu M, Parker LP, Koh DW. Inhibition of the transient receptor potential melastatin-2 channel causes increased DNA damage and decreased proliferation in breast adenocarcinoma cells. *Int J Oncol.* (2015) 46:2267–76. doi: 10.3892/ijo.2015.2919
62. Meng X, Cai C, Wu J, Cai S, Ye C, Chen H, et al. TRPM7 mediates breast cancer cell migration and invasion through the MAPK pathway. *Cancer Lett.* (2013) 333:96–102. doi: 10.1016/j.canlet.2013.01.031
63. Kuipers AJ, Middelbeek J, Vrenken K, Perez-Gonzalez C, Poelmans G, Klarenbeek J, et al. TRPM7 controls mesenchymal features of breast cancer cells by tensional regulation of SOX4. *Biochim Biophys Acta Mol Basis Dis.* (2018) 1864:2409–19. doi: 10.1016/j.bbadis.2018.04.017
64. Middelbeek J, Kuipers AJ, Henneman L, Visser D, Eidhof I, van Horssen R, et al. TRPM7 is required for breast tumor cell metastasis. *Cancer Res.* (2012) 72:4250–61. doi: 10.1158/0008-5472.CAN-11-3863
65. Guilbert A, Gautier M, Dhennin-Duthille I, Haren N, Sevestre H, Ouadid-Ahidouch H. Evidence that TRPM7 is required for breast cancer cell proliferation. *Am J Physiol Cell Physiol.* (2009) 297:C493–502. doi: 10.1152/ajpcell.00624.2008
66. Liu J, Chen Y, Shuai S, Ding D, Li R, Luo R. TRPM8 promotes aggressiveness of breast cancer cells by regulating EMT via activating AKT/GSK-3beta pathway. *Tum Biol.* (2014) 35:8969–77. doi: 10.1007/s13277-014-2077-8
67. Takahashi N, Chen HY, Harris IS, Stover DG, Selfors LM, Bronson RT, et al. Cancer cells co-opt the neuronal redox-sensing channel TRPA1 to promote oxidative-stress tolerance. *Cancer Cell.* (2018) 33:985–1003 e7. doi: 10.1016/j.ccell.2018.05.001
68. Chen J, Lu L, Feng Y, Wang H, Dai L, Li Y, et al. PKD2 mediates multi-drug resistance in breast cancer cells through modulation of P-glycoprotein expression. *Cancer Lett.* (2011) 300:48–56. doi: 10.1016/j.canlet.2010.09.005
69. Erkhembaatar M, Gu DR, Lee SH, Yang YM, Park S, Muallem S, et al. Lysosomal Ca(2+) signaling is essential for osteoclastogenesis and bone remodeling. *J Bone Miner Res.* (2017) 32:385–96. doi: 10.1002/jbmr.2986
70. Xu M, Almasi S, Yang Y, Yan C, Sterea AM, Rizvi Syeda AK, et al. The lysosomal TRPML1 channel regulates triple negative breast cancer development by promoting mTORC1 and purinergic signaling pathways. *Cell Calcium.* (2019) 79:80–8. doi: 10.1016/j.ceca.2019.02.010
71. Ji Y, Han Z, Shao L, Zhao Y. Ultrasound-targeted microbubble destruction of calcium channel subunit alpha 1D siRNA inhibits breast cancer via G protein-coupled receptor 30. *Oncol Rep.* (2016) 36:1886–92. doi: 10.3892/or.2016.5031
72. Ohkubo T, Yamazaki J. T-type voltage-activated calcium channel Cav3.1, but not Cav3.2, is involved in the inhibition of proliferation and apoptosis in MCF-7 human breast cancer cells. *Int J Oncol.* (2012) 41:267–75. doi: 10.3892/ijo.2012.1422
73. Pera E, Kaemmerer E, Milevskiy MJG, Yapa K, O'Donnell JS, Brown MA, et al. The voltage gated Ca(2+)-channel Cav3.2 and therapeutic responses in breast cancer. *Cancer Cell Int.* (2016) 16:24. doi: 10.1186/s12935-016-0299-0
74. Basson MD, Zeng B, Downey C, Sirivelu MP, Tepe JJ. Increased extracellular pressure stimulates tumor proliferation by a mechanosensitive calcium channel and PKC-beta. *Mol Oncol.* (2015) 9:513–26. doi: 10.1016/j.molonc.2014.10.008
75. Jardin I, Lopez JJ, Salido GM, Rosado JA. Store-Operated Ca(2+) entry in breast cancer cells: remodeling and functional role. *Int J Mol Sci.* (2018) 19:4053. doi: 10.3390/ijms19124053
76. Yang S, Zhang JJ, Huang XY. Orai1 and STIM1 are critical for breast tumor cell migration and metastasis. *Cancer Cell.* (2009) 15:124–34. doi: 10.1016/j.ccr.2008.12.019
77. Didiasova M, Zakrzewicz D, Magdolen V, Nagaraj C, Balint Z, Rohde M, et al. STIM1/ORAI1-mediated Ca2+ influx regulates enolase-1 exteriorization. *J Biol Chem.* (2015) 290:11983–99. doi: 10.1074/jbc.M114.598425
78. Zhang S, Miao Y, Zheng X, Gong Y, Zhang J, Zou F, et al. STIM1 and STIM2 differently regulate endogenous Ca(2+) entry and promote TGF-beta-induced EMT in breast cancer cells. *Biochem Biophys Res Commun.* (2017) 488:74–80. doi: 10.1016/j.bbrc.2017.05.009
79. Cheng H, Wang S, Feng R. STIM1 plays an important role in TGF-beta-induced suppression of breast cancer cell proliferation. *Oncotarget.* (2016) 7:16866–78. doi: 10.18632/oncotarget.7619
80. Kim MS, Yang YM, Son A, Tian YS, Lee SI, Kang SW, et al. RANKL-mediated reactive oxygen species pathway that induces long lasting Ca2+ oscillations essential for osteoclastogenesis. *J Biol Chem.* (2010) 285:6913–21. doi: 10.1074/jbc.M109.051557
81. Robinson LJ, Mancarella S, Songsawad D, Tourkova IL, Barnett JB, Gill DL, et al. Gene disruption of the calcium channel Orai1 results in inhibition of osteoclast and osteoblast differentiation and impairs skeletal development. *Lab Invest.* (2012) 92:1071–83. doi: 10.1038/labinvest.2012.72
82. Hwang SY, Putney JW. Orai1-mediated calcium entry plays a critical role in osteoclast differentiation and function by regulating activation of the transcription factor NFATc1. *FASEB J.* (2012) 26:1484–92. doi: 10.1096/fj.11-194399
83. Hwang SY, Foley J, Numaga-Tomita T, Petranka JG, Bird GS, Putney JW, Jr. Deletion of Orai1 alters expression of multiple genes during osteoclast and osteoblast maturation. *Cell Calcium.* (2012) 52:488–500. doi: 10.1016/j.ceca.2012.10.001
84. Chantome A, Potier-Cartereau M, Clarysse L, Fromont G, Marionneau-Lambot S, Gueguinou M, et al. Pivotal role of the lipid Raft SK3-Orai1 complex in human cancer cell migration and bone metastases. *Cancer Res.* (2013) 73:4852–61. doi: 10.1158/0008-5472.CAN-12-4572
85. Xia J, Yu X, Tang L, Li G, He T. P2X7 receptor stimulates breast cancer cell invasion and migration via the AKT pathway. *Oncol Rep.* (2015) 34:103–10. doi: 10.3892/or.2015.3979
86. Jelassi B, Chantome A, Alcaraz-Perez F, Baroja-Mazo A, Cayuela ML, Pelegrin P, et al. P2X(7) receptor activation enhances SK3 channels- and cystein cathepsin-dependent cancer cells invasiveness. *Oncogene.* (2011) 30:2108–22. doi: 10.1038/onc.2010.593
87. Park M, Kim J, Phuong NTT, Park JG, Park JH, Kim YC, et al. Involvement of the P2X7 receptor in the migration and metastasis of tamoxifen-resistant breast cancer: effects on small extracellular vesicles production. *Scient Rep.* (2019) 9:11587. doi: 10.1038/s41598-019-47734-z
88. Tan C, Han LI, Zou L, Luo C, Liu A, Sheng X, et al. Expression of P2X7R in breast cancer tissue and the induction of apoptosis by the gene-specific shRNA in MCF-7 cells. *Exp Therap Med.* (2015) 10:1472–8. doi: 10.3892/etm.2015.2705
89. Gilbert SM, Oliphant CJ, Hassan S, Peille AL, Bronsert P, Falzoni S, et al. ATP in the tumour microenvironment drives expression of nfP2X7, a key mediator of cancer cell survival. *Oncogene.* (2019) 38:194–208. doi: 10.1038/s41388-018-0426-6
90. Korcok J, Raimundo LN, Ke HZ, Sims SM, Dixon SJ. Extracellular nucleotides act through P2X7 receptors to activate NF-kappaB in osteoclasts. *J Bone Miner Res.* (2004) 19:642–51. doi: 10.1359/JBMR.040108
91. Gartland A, Buckley KA, Hipskind RA, Perry MJ, Tobias JH, Buell G, et al. Multinucleated osteoclast formation *in vivo* and *in vitro* by P2X7 receptor-deficient mice. *Crit Rev Eukary Gene Express.* (2003) 13:243–53. doi: 10.1615/CritRevEukaryotGeneExpr.v13.i24.160
92. Wang N, Agrawal A, Jorgensen NR, Gartland A. P2X7 receptor regulates osteoclast function and bone loss in a mouse model of osteoporosis. *Scient Rep.* (2018) 8:3507. doi: 10.1038/s41598-018-21574-9
93. Pellegatti P, Falzoni S, Donvito G, Lemaire I, Di Virgilio F. P2X7 receptor drives osteoclast fusion by increasing the extracellular adenosine concentration. *FASEB J.* (2011) 25:1264–74. doi: 10.1096/fj.10-169854

94. Agrawal A, Buckley KA, Bowers K, Furber M, Gallagher JA, Gartland A. The effects of P2X7 receptor antagonists on the formation and function of human osteoclasts *in vitro*. *Puriner Signal.* (2010) 6:307–15. doi: 10.1007/s11302-010-9181-z
95. Zhou JZ, Riquelme MA, Gao X, Ellies LG, Sun LZ, Jiang JX. Differential impact of adenosine nucleotides released by osteocytes on breast cancer growth and bone metastasis. *Oncogene.* (2015) 34:1831–42. doi: 10.1038/onc.2014.113
96. Venkatachalam K, Montell C. TRP channels. *Ann Rev Biochem.* (2007) 76:387–417. doi: 10.1146/annurev.biochem.75.103004.142819
97. Rohacs T. Teaching resources. TRP channels. Science's STKE: signal transduction knowledge environment. *Eur PMC.* (2005) 2005:tr14. doi: 10.1126/stke.2822005tr14
98. Vangeel L, Voets T. Transient receptor potential channels and calcium signaling. *Cold Spring Harbor Perspect Biol.* (2019) 11:a035048. doi: 10.1101/cshperspect.a035048
99. Rohacs T, Thyagarajan B, Lukacs V. Phospholipase C mediated modulation of TRPV1 channels. *Mol Neurobiol.* (2008) 37:153–63. doi: 10.1007/s12035-008-8027-y
100. Gautier M, Dhennin-Duthille I, Ay AS, Rybarczyk P, Korichneva I, Ouadid-Ahidouch H. New insights into pharmacological tools to TR(i)P cancer up. *Br J Pharmacol.* (2014) 171:2582–92. doi: 10.1111/bph.12561
101. Dhennin-Duthille I, Gautier M, Faouzi M, Guilbert A, Brevet M, Vaudry D, et al. High expression of transient receptor potential channels in human breast cancer epithelial cells and tissues: correlation with pathological parameters. *Cell Physiol Biochem.* (2011) 28:813–22. doi: 10.1159/000335795
102. Lastraioli E, Iorio J, Arcangeli A. Ion channel expression as promising cancer biomarker. *Biochim Biophys Acta.* (2015) 1848(10 Pt B):2685–702. doi: 10.1016/j.bbamem.2014.12.016
103. Rosenbaum T, Simon SA. TRPV1 receptors and signal transduction. In: Liedtke WB, Heller S, editors. *TRP Ion Channel Function in Sensory Transduction and Cellular Signaling Cascades*. Boca Raton, FL: Frontiers in Neuroscience (2007). doi: 10.1201/9781420005844.ch5
104. Fornetti J, Welm AL, Stewart SA. Understanding the Bone in Cancer Metastasis. *J Bone Mineral Res.* (2018) 33:2099–113. doi: 10.1002/jbmr.3618
105. Lotinun S, Kiviranta R, Matsubara T, Alzate JA, Neff L, Luth A, et al. Osteoclast-specific cathepsin K deletion stimulates S1P-dependent bone formation. *J Clin Invest.* (2013) 123:666–81. doi: 10.1172/JCI64840
106. Hantute-Ghesquier A, Haustrate A, Prevarskaya N, Lehen'kyi V. TRPM family channels in cancer. *Pharmaceuticals.* (2018) 11:58. doi: 10.3390/ph11020058
107. Ishii M, Hagiwara T, Mori Y, Shimizu S. Involvement of TRPM2 and L-type Ca(2)(+) channels in Ca(2)(+) entry and cell death induced by hydrogen peroxide in rat beta-cell line RIN-5F. *J Toxicol Sci.* (2014) 39:199–209. doi: 10.2131/jts.39.199
108. Kheradpezhouh E, Ma L, Morphett A, Barritt GJ, Rychkov GY. TRPM2 channels mediate acetaminophen-induced liver damage. *Proc Natl Acad Sci USA.* (2014) 111:3176–81. doi: 10.1073/pnas.1322657111
109. Liu X, Cotrim A, Teos L, Zheng C, Swaim W, Mitchell J, et al. Loss of TRPM2 function protects against irradiation-induced salivary gland dysfunction. *Nat Commun.* (2013) 4:1515. doi: 10.1038/ncomms2526
110. Davis FM, Azimi I, Faville RA, Peters AA, Jalink K, Putney JW, Jr., et al. Induction of epithelial-mesenchymal transition (EMT) in breast cancer cells is calcium signal dependent. *Oncogene.* (2014) 33:2307–16. doi: 10.1038/onc.2013.187
111. Shen B, Sun L, Zheng H, Yang D, Zhang J, Zhang Q. The association between single-nucleotide polymorphisms of TRPM7 gene and breast cancer in Han Population of Northeast China. *Med Oncol.* (2014) 31:51. doi: 10.1007/s12032-014-0051-3
112. Guilbert A, Gautier M, Dhennin-Duthille I, Rybarczyk P, Sahni J, Sevestre H, et al. Transient receptor potential melastatin 7 is involved in oestrogen receptor-negative metastatic breast cancer cells migration through its kinase domain. *Eur J Cancer.* (2013) 49:3694–707. doi: 10.1016/j.ejca.2013.07.008
113. LaPlante JM, Falardeau J, Sun M, Kanazirska M, Brown EM, Slaugenhaupt SA, et al. Identification and characterization of the single channel function of human mucolipin-1 implicated in mucolipidosis type IV, a disorder affecting the lysosomal pathway. *FEBS Lett.* (2002) 532:183–7. doi: 10.1016/S0014-5793(02)03670-0
114. Wong YC, Kim S, Peng W, Krainc D. Regulation and function of mitochondria-lysosome membrane contact sites in cellular homeostasis. *Trends Cell Biol.* (2019) 29:500–13. doi: 10.1016/j.tcb.2019.02.004
115. Dolphin AC. Voltage-gated calcium channels and their auxiliary subunits: physiology and pathophysiology and pharmacology. *J Physiol.* (2016) 594:5369–90. doi: 10.1113/JP272262
116. Kaestner L, Wang X, Hertz L, Bernhardt I. Voltage-activated ion channels in non-excitable cells-A viewpoint regarding their physiological justification. *Front Physiol.* (2018) 9:450. doi: 10.3389/fphys.2018.00450
117. Catterall WA. Voltage-gated calcium channels. *Cold Spring Harbor Perspect Biol.* (2011) 3:a003947. doi: 10.1101/cshperspect.a003947
118. Yu HG, McLaughlin S, Newman M, Brundage K, Ammer A, Martin K, et al. Altering calcium influx for selective destruction of breast tumor. *BMC Cancer.* (2017) 17:169. doi: 10.1186/s12885-017-3168-x
119. Bhargava A, Saha S. T-Type voltage gated calcium channels: a target in breast cancer? Breast cancer research and treatment. *Breast Cancer Res Treat*. (2019) 173:11–21. doi: 10.1007/s10549-018-4970-0
120. Lewis RS. Store-operated calcium channels: new perspectives on mechanism and function. *Cold Spring Harbor Persp Biol.* (2011) 3:a003970. doi: 10.1101/cshperspect.a003970
121. Roos J, DiGregorio PJ, Yeromin AV, Ohlsen K, Lioudyno M, Zhang S, et al. STIM1, an essential and conserved component of store-operated Ca2+ channel function. *J Cell Biol.* (2005) 169:435–45. doi: 10.1083/jcb.200502019
122. Liou J, Kim ML, Heo WD, Jones JT, Myers JW, Ferrell JE, Jr., et al. STIM is a Ca2+ sensor essential for Ca2+-store-depletion-triggered Ca2+ influx. *Curr Biol.* (2005) 15:1235–41. doi: 10.1016/j.cub.2005.05.055
123. Zhang SL, Yu Y, Roos J, Kozak JA, Deerinck TJ, Ellisman MH, et al. STIM1 is a Ca2+ sensor that activates CRAC channels and migrates from the Ca2+ store to the plasma membrane. *Nature.* (2005) 437:902–5. doi: 10.1038/nature04147
124. Prakriya M, Feske S, Gwack Y, Srikanth S, Rao A, Hogan PG. Orai1 is an essential pore subunit of the CRAC channel. *Nature.* (2006) 443:230–3. doi: 10.1038/nature05122
125. Vig M, Peinelt C, Beck A, Koomoa DL, Rabah D, Koblan-Huberson M, et al. CRACM1 is a plasma membrane protein essential for store-operated Ca2+ entry. *Science.* (2006) 312:1220–3. doi: 10.1126/science.1127883
126. Zhang SL, Yeromin AV, Zhang XH, Yu Y, Safrina O, Penna A, et al. Genome-wide RNAi screen of Ca(2+) influx identifies genes that regulate Ca(2+) release-activated Ca(2+) channel activity. *Proc Natl Acad Sci USA.* (2006) 103:9357–62. doi: 10.1073/pnas.0603161103
127. Feske S, Skolnik EY, Prakriya M. Ion channels and transporters in lymphocyte function and immunity. *Nat Rev Immunol.* (2012) 12:532–47. doi: 10.1038/nri3233
128. Lacruz RS, Feske S. Diseases caused by mutations in ORAI1 and STIM1. *Ann NY Acad Sci.* (2015) 1356:45–79. doi: 10.1111/nyas.12938
129. Wygrecka M, Marsh LM, Morty RE, Henneke I, Guenther A, Lohmeyer J, et al. Enolase-1 promotes plasminogen-mediated recruitment of monocytes to the acutely inflamed lung. *Blood.* (2009) 113:5588–98. doi: 10.1182/blood-2008-08-170837
130. Massague J. TGFbeta in cancer. *Cell.* (2008) 134:215–30. doi: 10.1016/j.cell.2008.07.001
131. Yang Y, Jiang Z, Wang B, Chang L, Liu J, Zhang L, et al. Expression of STIM1 is associated with tumor aggressiveness and poor prognosis in breast cancer. *Pathol Res Practice.* (2017) 213:1043–7. doi: 10.1016/j.prp.2017.08.006
132. Ou-Yang F, Lin YD, Chuang LY, Chang HW, Yang CH, Hou MF. The combinational polymorphisms of ORAI1 gene are associated with preventive models of breast cancer in the Taiwanese. *BioMed Res Int.* (2015) 2015:281263. doi: 10.1155/2015/281263
133. Chang WC, Woon PY, Hsu YW, Yang S, Chiu YC, Hou MF. The association between single-nucleotide polymorphisms of ORAI1 gene and breast cancer in a Taiwanese population. *Scient World J.* (2012) 2012:916587. doi: 10.1100/2012/916587
134. Chang WC, Fang YY, Chang HW, Chuang LY, Lin YD, Hou MF, et al. Identifying association model for single-nucleotide polymorphisms of ORAI1 gene for breast cancer. *Cancer Cell Int.* (2014) 14:29. doi: 10.1186/1475-2867-14-29
135. Zhang Z, Xu Q, Song C, Mi B, Zhang H, Kang H, et al. Serum- and glucocorticoid-inducible kinase 1 is essential for osteoclastogenesis and

promotes breast cancer bone metastasis. *Mol Cancer Therap.* (2020) 19:650–660. doi: 10.1158/1535-7163.MCT-18-0783
136. Jing J, He L, Sun A, Quintana A, Ding Y, Ma G, et al. Proteomic mapping of ER-PM junctions identifies STIMATE as a regulator of Ca(2)(+) influx. *Nat Cell Biol.* (2015) 17:1339–47. doi: 10.1038/ncb3234
137. Quintana A, Rajanikanth V, Farber-Katz S, Gudlur A, Zhang C, Jing J, et al. TMEM110 regulates the maintenance and remodeling of mammalian ER-plasma membrane junctions competent for STIM-ORAI signaling. *Proc Natl Acad Sci USA.* (2015) 112:E7083–92. doi: 10.1073/pnas.1521924112
138. Palty R, Raveh A, Kaminsky I, Meller R, Reuveny E. SARAF inactivates the store operated calcium entry machinery to prevent excess calcium refilling. *Cell.* (2012) 149:425–38. doi: 10.1016/j.cell.2012.01.055
139. Krapivinsky G, Krapivinsky L, Stotz SC, Manasian Y, Clapham DE. POST, partner of stromal interaction molecule 1 (STIM1), targets STIM1 to multiple transporters. *Proc Natl Acad Sci USA.* (2011) 108:19234–9. doi: 10.1073/pnas.1117231108
140. Yang Z, Yan H, Dai W, Jing J, Yang Y, Mahajan S, et al. Tmem178 negatively regulates store-operated calcium entry in myeloid cells via association with STIM1. *J Autoimmun.* (2019) 101:94–108. doi: 10.1016/j.jaut.2019.04.015
141. Srikanth S, Woo JS, Wu B, El-Sherbiny YM, Leung J, Chupradit K, et al. The Ca(2+) sensor STIM1 regulates the type I interferon response by retaining the signaling adaptor STING at the endoplasmic reticulum. *Nat Immunol.* (2019) 20:152–62. doi: 10.1038/s41590-018-0287-8
142. Wang QC, Wang X, Tang TS. EB1 traps STIM1 and regulates local store-operated Ca(2+) entry. *J Cell Biol.* (2018) 217:1899–900. doi: 10.1083/jcb.201805037
143. Chen YJ, Chang CL, Lee WR, Liou J. RASSF4 controls SOCE and ER-PM junctions through regulation of PI(4,5)P2. *J Cell Biol.* (2017) 216:2011–25. doi: 10.1083/jcb.201606047
144. Srivats S, Balasuriya D, Pasche M, Vistal G, Edwardson JM, Taylor CW, et al. Sigma1 receptors inhibit store-operated Ca2+ entry by attenuating coupling of STIM1 to Orai1. *J Cell Biol.* (2016) 213:65–79. doi: 10.1083/jcb.201506022
145. Khakh BS, North RA. P2X receptors as cell-surface ATP sensors in health and disease. *Nature.* (2006) 442:527–32. doi: 10.1038/nature04886
146. Wang H, Gong C, Liu X, Rao S, Li T, He L, et al. Genetic interaction of purinergic P2X7 receptor and ER-alpha polymorphisms in susceptibility to osteoporosis in Chinese postmenopausal women. *J Bone Miner Metab.* (2018) 36:488–97. doi: 10.1007/s00774-017-0862-3
147. Jorgensen NR, Husted LB, Skarratt KK, Stokes L, Tofteng CL, Kvist T, et al. Single-nucleotide polymorphisms in the P2X7 receptor gene are associated with post-menopausal bone loss and vertebral fractures. *Eur J Hum Genet.* (2012) 20:675–81. doi: 10.1038/ejhg.2011.253
148. Ohlendorff SD, Tofteng CL, Jensen JE, Petersen S, Civitelli R, Fenger M, et al. Single nucleotide polymorphisms in the P2X7 gene are associated to fracture risk and to effect of estrogen treatment. *Pharmacogenet Genomics.* (2007) 17:555–67. doi: 10.1097/FPC.0b013e3280951625
149. Suzuki T, Notomi T, Miyajima D, Mizoguchi F, Hayata T, Nakamoto T, et al. Osteoblastic differentiation enhances expression of TRPV4 that is required for calcium oscillation induced by mechanical force. *Bone.* (2013) 54:172–8. doi: 10.1016/j.bone.2013.01.001
150. Jing D, Baik AD, Lu XL, Zhou B, Lai X, Wang L, et al. In situ intracellular calcium oscillations in osteocytes in intact mouse long bones under dynamic mechanical loading. *FASEB J.* (2014) 28:1582–92. doi: 10.1096/fj.13-237578
151. Abed E, Moreau R. Importance of melastatin-like transient receptor potential 7 and cations (magnesium, calcium) in human osteoblast-like cell proliferation. *Cell Prolifer.* (2007) 40:849–65. doi: 10.1111/j.1365-2184.2007.00476.x
152. Abed E, Martineau C, Moreau R. Role of melastatin transient receptor potential 7 channels in the osteoblastic differentiation of murine MC3T3 cells. *Calcif Tissue Int.* (2011) 88:246–53. doi: 10.1007/s00223-010-9455-z
153. Xiao Z, Cao L, Liang Y, Huang J, Stern AR, Dallas M, et al. Osteoblast-specific deletion of Pkd2 leads to low-turnover osteopenia and reduced bone marrow adiposity. *PLoS ONE.* (2014) 9:e114198. doi: 10.1371/journal.pone.0114198
154. Cao C, Ren Y, Barnett AS, Mirando AJ, Rouse D, Mun SH, et al. Increased Ca2+ signaling through CaV1.2 promotes bone formation and prevents estrogen deficiency-induced bone loss. *JCI Insight.* (2017) 2:e95512. doi: 10.1172/jci.insight.95512
155. Lee SH, Park Y, Song M, Srikanth S, Kim S, Kang MK, et al. Orai1 mediates osteogenic differentiation via BMP signaling pathway in bone marrow mesenchymal stem cells. *Biochem Biophys Res Commun.* (2016) 473:1309–14. doi: 10.1016/j.bbrc.2016.04.068
156. Choi H, Srikanth S, Atti E, Pirih FQ, Nervina JM, Gwack Y, et al. Deletion of Orai1 leads to bone loss aggravated with aging and impairs function of osteoblast lineage cells. *Bone Rep.* (2018) 8:147–55. doi: 10.1016/j.bonr.2018.03.007
157. Sindhavajiva PR, Sastravaha P, Arksornnukit M, Pavasant P. Intermittent compressive force induces human mandibular-derived osteoblast differentiation via WNT/beta-catenin signaling. *J Cell Biochem.* (2018) 119:3474–85. doi: 10.1002/jcb.26519
158. Feng Y, Zhou M, Zhang Q, Liu H, Xu Y, Shu L, et al. Synergistic effects of high dietary calcium and exogenous parathyroid hormone in promoting osteoblastic bone formation in mice. *Br J Nutr.* (2015) 113:909–22. doi: 10.1017/S0007114514004309
159. Sooampon S, Manokawinchoke J, Pavasant P. Transient receptor potential vanilloid-1 regulates osteoprotegerin/RANKL homeostasis in human periodontal ligament cells. *J Periodontal Res.* (2013) 48:22–9. doi: 10.1111/j.1600-0765.2012.01493.x
160. Kennecke H, Yerushalmi R, Woods R, Cheang MC, Voduc D, Speers CH, et al. Metastatic behavior of breast cancer subtypes. *J Clin Oncol.* (2010) 28:3271–7. doi: 10.1200/JCO.2009.25.9820
161. Cancer Genome Atlas N. Comprehensive molecular portraits of human breast tumours. *Nature.* (2012) 490:61–70. doi: 10.1038/nature11412
162. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. *Nature.* (2000) 406:747–52. doi: 10.1038/35021093
163. Makena MR, Rao R. Subtype specific targeting of calcium signaling in breast cancer. *Cell Calcium.* (2020) 85:102109. doi: 10.1016/j.ceca.2019.102109
164. So CL, Saunus JM, Roberts-Thomson SJ, Monteith GR. Calcium signalling and breast cancer. *Sem Cell Dev Biol.* (2019) 94:74–83. doi: 10.1016/j.semcdb.2018.11.001
165. Magby JP, Richardson JR. Role of calcium and calpain in the downregulation of voltage-gated sodium channel expression by the pyrethroid pesticide deltamethrin. *J Biochem Mol Toxicol.* (2015) 29:129–34. doi: 10.1002/jbt.21676
166. Azimi I, Roberts-Thomson SJ, Monteith GR. Calcium influx pathways in breast cancer: opportunities for pharmacological intervention. *Br J Pharmacol.* (2014) 171:945–60. doi: 10.1111/bph.12486
167. Drake MT, Clarke BL, Khosla S. Bisphosphonates: mechanism of action and role in clinical practice. *Mayo Clin Proc.* (2008) 83:1032–45. doi: 10.4065/83.9.1032
168. Vasikaran SD. Bisphosphonates: an overview with special reference to alendronate. *Ann Clin Biochem.* (2001) 38(Pt 6):608–23. doi: 10.1258/0004563011901037
169. Roelofs AJ, Thompson K, Gordon S, Rogers MJ. Molecular mechanisms of action of bisphosphonates: current status. *Clin Cancer Res.* (2006) 12(20 Pt 2):6222s−30s. doi: 10.1158/1078-0432.CCR-06-0843
170. Chesnut CH III, Harris ST. Short-term effect of alendronate on bone mass and bone remodeling in postmenopausal women. *Osteoporosis Int.* (1993) 3 (Suppl. 3):S17–9. doi: 10.1007/BF01623003
171. Kostenuik PJ, Nguyen HQ, McCabe J, Warmington KS, Kurahara C, Sun N, et al. Denosumab, a fully human monoclonal antibody to RANKL, inhibits bone resorption and increases BMD in knock-in mice that express chimeric (murine/human) RANKL. *J Bone Mineral Res.* (2009) 24:182–95. doi: 10.1359/jbmr.081112
172. Lacey DL, Boyle WJ, Simonet WS, Kostenuik PJ, Dougall WC, Sullivan JK, et al. Bench to bedside: elucidation of the OPG-RANK-RANKL pathway and the development of denosumab. *Nat Rev Drug Disc.* (2012) 11:401–19. doi: 10.1038/nrd3705

173. Chawla S, Henshaw R, Seeger L, Choy E, Blay JY, Ferrari S, et al. Safety and efficacy of denosumab for adults and skeletally mature adolescents with giant cell tumour of bone: interim analysis of an open-label, parallel-group, phase 2 study. *Lancet Oncol.* (2013) 14:901–8. doi: 10.1016/S1470-2045(13)70277-8
174. Granholm S, Lundberg P, Lerner UH. Calcitonin inhibits osteoclast formation in mouse haematopoetic cells independently of transcriptional regulation by receptor activator of NF-{kappa}B and c-Fms. *J Endocrinol.* (2007) 195:415–27. doi: 10.1677/JOE-07-0338
175. Feldenzer KL, Sarno J. Hypercalcemia of malignancy. *J Adv Pract Oncol.* (2018) 9:496–504. doi: 10.6004/jadpro.2018.9.5.4

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REVIEW
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The Endosteal Niche in Breast Cancer Bone Metastasis

Marie-Therese Haider[1], *Daniel J. Smit*[2] *and Hanna Taipaleenmäki*[1]*

[1] *Molecular Skeletal Biology Laboratory, Department of Trauma, Hand and Reconstructive Surgery, University Medical Center Hamburg-Eppendorf, Hamburg, Germany,* [2] *Institute of Biochemistry and Signal Transduction, University Medical Center Hamburg-Eppendorf, Hamburg, Germany*

The establishment of bone metastasis remains one of the most frequent complications of patients suffering from advanced breast cancer. Patients with bone metastases experience high morbidity and mortality caused by excessive, tumor-induced and osteoclast-mediated bone resorption. Anti-resorptive treatments, such as bisphosphonates, are available to ease skeletal related events including pain, increased fracture risk, and hypercalcemia. However, the disease remains incurable and 5-year survival rates for these patients are below 25%. Within the bone, disseminated breast cancer cells localize in "metastatic niches," special microenvironments that are thought to regulate cancer cell colonization and dormancy as well as tumor progression and subsequent development into overt metastases. Precise location and composition of this "metastatic niche" remain poorly defined. However, it is thought to include an "endosteal niche" that is composed of key bone cells that are derived from both, hematopoietic stem cells (osteoclasts), and mesenchymal stromal cells (osteoblasts, fibroblasts, adipocytes). Our knowledge of how osteoclasts drive the late stage of the disease is well-established. In contrast, much less is known about the interaction between osteogenic cells and disseminated tumor cells prior to the initiation of the osteolytic phase. Recent studies suggest that mesenchymal-derived cells, including osteoblasts and fibroblasts, play a key role during the early stages of breast cancer bone metastasis such as tumor cell homing, bone marrow colonization, and tumor cell dormancy. Hence, elucidating the interactions between breast cancer cells and mesenchymal-derived cells that drive metastasis progression could provide novel therapeutic approaches and targets to treat breast cancer bone metastasis. In this review we discuss evidences reporting the interaction between tumor cells and endosteal niche cells during the early stages of breast cancer bone metastasis, with a particular focus on mesenchymal-derived osteoblasts and fibroblasts.

Keywords: breast cancer, bone metastases, endosteal niche, microenvironment, osteoblast, fibroblast

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Monash University, Australia

***Correspondence:**
Hanna Taipaleenmäki
h.taipaleenmaeki@uke.de

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INTRODUCTION

Metastasis is a complex, multi-step process during which cancer cells escape from the primary tumor, circulate, disseminate to the distant organs, and eventually colonize and grow in the metastatic site (1). One of the essential steps in metastases development is the ability of cancer cells to adapt to the new environment which is very different from the environment in the tissue of origin. The interaction between cancer cells and the metastatic environment was already proposed

in 1889 by Sir Stephen Paget who suggested that metastatic colonization of a distant organ is not a random process and that cancer cells can only grow in a supportive microenvironment (2). This so called "Seed and Soil" theory in which the cancer cells are the seeds and the bone is the soil can be considered as the first evidence of the "niche" concept. Nevertheless, more than a century later we are still in process of understanding the complex interaction between the cancer cells and the local and metastatic microenvironments or "niches."

Bone metastases involve complex interactions between the cancer cells and the cells of the bone microenvironment, including endothelial cells, hematopoietic stem cells (HSCs), mesenchymal stromal cells (MSCs), and bone cells (bone forming osteoblasts and bone resorbing osteoclasts) (3). The role of osteoclasts in driving the progression of breast cancer bone metastases is well-established (4). During the so called "vicious cycle of bone metastases" osteoclasts are activated directly or indirectly by the tumor cells (5). Increased osteoclast function results in pathological bone resorption during which several growth factors, including transforming growth factor β (TGF-β) are released from the bone matrix. These factors support tumor growth and further osteoclast activation (6). In contrast, the contribution of osteoblasts to disease establishment has been underappreciated and poorly investigated. However, recently research has moved away from the concept that osteoclasts alone drive the progression of breast cancer bone metastasis and osteoblasts are more and more investigated as novel cellular targets (7, 8). In order to develop novel, more successful therapies to prevent or treat cancer-induced bone disease, a better understanding of the interaction of tumor cells and cells of the bone microenvironment is required, in particular the tumor—bone cell communications prior to the formation of osteolytic lesions. In the following chapters we will discuss the role of bone marrow niches, in particular the endosteal niche, in the development and progression of bone metastasis as well as the function of osteoblasts and fibroblasts in this process.

BONE MARROW NICHES

Physiological Niche

In bone, the physiological niche is composed of several local environments including the endosteal niche and the vascular niche (9). The endosteal niche lines the trabecular and endocortical bone surface and consists of osteoblasts that form new bone and osteoclasts that resorb the bone. Osteoblasts are derived from MSCs in a process tightly controlled by various transcription factors and signaling pathways. The key transcription factors Runx2 and osterix (Osx) promote MSC commitment to osteoprogenitors and further differentiation to mature osteoblasts (10). Mature osteoblasts secrete bone matrix proteins including collagen I (Col1), alkaline phosphatase (ALP) and osteocalcin, and contribute to bone formation. Mature osteoblasts can be embedded in the bone matrix as osteocytes that function as mechanosensory cells and contribute to bone remodeling (11). Alternatively, osteoblasts can adapt a quiescent state on the bone surface as bone lining cells or undergo apoptosis. Osteoblast differentiation is promoted by various paracrine factors including parathyroid hormone (PTH) and wingless (Wnt) proteins that activate the respective signaling pathways (11, 12). Besides osteoblasts, MSCs can give rise to other mesenchymal cell populations including adipocytes, chondrocytes and myocytes. Adipocytes are a frequent cell type in the bone marrow and an inverse relationship has been shown to occur between osteogenesis and adipogenesis of MSCs (13).

Bone-resorbing osteoclasts are multinucleated cells of hematopoietic origin. Osteoclast differentiation is supported by various cytokines including the macrophage colony-stimulating factor (MCS-F) and the receptor activator of nuclear factor kappa-B ligand (RANKL) that are produced by osteoblasts (14). In turn, osteoclasts secrete factors such as Wnt 10b, sphingosine-1-phosphate and bone morphogenic protein 6 (BMP-6) to regulate osteoblast differentiation and function. Additionally, bone matrix-derived factors including but not limited to TGF-ß, insulin like growth factors (IGFs) and bone morphogenic proteins (BMPs) are released during osteoclast-mediated bone resorption and can modify osteoblast progenitors. Detailed coupling mechanisms between osteoblasts and osteoclasts are reviewed in (15). Through these coordinated actions bone formation and resorption are often coupled under physiological conditions. In addition to its role in bone remodeling, the endosteal niche has been proposed to maintain hematopoietic stem cells (HSCs) in a quiescent state.

The vascular niche consists of endothelial cells, closely located pericytes and smooth muscle cells. The vascular niche is important for stem and progenitor cell function. Through secretion of angiocrine growth factors, the vascular niche recruits endothelial progenitors, MSCs and HSCs (16, 17). In contrast to the endosteal niche that supports HSC quiescence, the vascular niche has been shown to promote HSC mobilization, proliferation and differentiation and thus the activation of HSCs (17). Although the endosteal and vascular niches can be considered as independent microenvironments their interaction is crucial for various physiological functions including HSC maintenance and coupling of angiogenesis and osteogenesis (17, 18).

Pre-metastatic Niche

In cancer, the physiological functions of the niches are hijacked by metastatic cancer cells. Cancer cells alter the niche to support their own functions from tumor cell dissemination to dormancy, relapse, and growth. Importantly, the first changes in the expression of the components of the extracellular matrix (ECM) and mobilization of bone marrow progenitor cells occur already before the cancer cells arrive in the metastatic site such as the bone marrow or the lung (19). Preparation of this so-called "pre-metastatic niche" creates a conductive microenvironment for the cancer cells that eventually disseminate to distant organs.

Formation of the pre-metastatic niche requires remodeling of the ECM and deposition of aberrant ECM. Important ECM proteins include fibronectin, tenascin and periostin that form fibrillar networks and regulate cancer cell adhesion and growth (19, 20). Among other factors, breast cancer cells in the primary tumor secrete lysyl oxidase (LOX) that regulates fibronectin activity and matrix remodeling (21). LOX also alters the endosteal

niche by activating the osteoclasts, thus preparing a permissive environment for circulating tumor cells to colonize the bone (22). Interestingly, high LOX expression in the primary tumor is associated with bone metastases without affecting the primary tumor growth. Recently, tumor exosomes have been shown to prepare the pre-metastatic niche and direct organotrophic metastasis through the expression of diverse integrins (23).

Metastatic Niche

Within bone, the proposed metastatic niche is composed of several individual and distinct cellular entities comprising a hematopoietic, endosteal, and vascular niche (**Figure 1**). Emerging evidence also implicates a role for the bone marrow adipocyte niche in bone metastasis (24, 25). These niches are thought to determine the fate of disseminated tumor cells (DTCs), namely whether they will actively proliferate, stay quiescent/dormant or die. Breast cancer dissemination to the bone has been shown to occur E-selectin-mediated interactions in the sinusoidal regions (vascular niche) (26). The sinusoidal vasculature also regulates HSC transit through the same mechanism and once in the bone, cancer cells have been proposed to compete with HSCs for their niche.

Both the vascular and the endosteal niche have been shown to maintain breast cancer cell dormancy though different cues (27, 28). The vascular niche has been proposed to function as a "pro-dormancy" niche maintaining the cancer cells quiescent. The niche-derived molecules regulating cancer cell dormancy include a chemokine stromal cell-derived factor 1 [SDF-1, also known as C-X-C motif chemokine ligand 12 (CXCL12)] that binds to its receptor C-X-C chemokine receptor type 4 (CXCR4) on cancer cells and anchors the cancer cells in the niche (26). In addition, thrombospondin expressed by endothelial cells in a stable microvasculature has been shown to induce breast cancer cell quiescence (27). While a stable vasculature promotes dormancy, active sprouting neovasculature has been proposed to release cancer cells from the dormant state and support micrometastases growth via TGF-β and periostin (27).

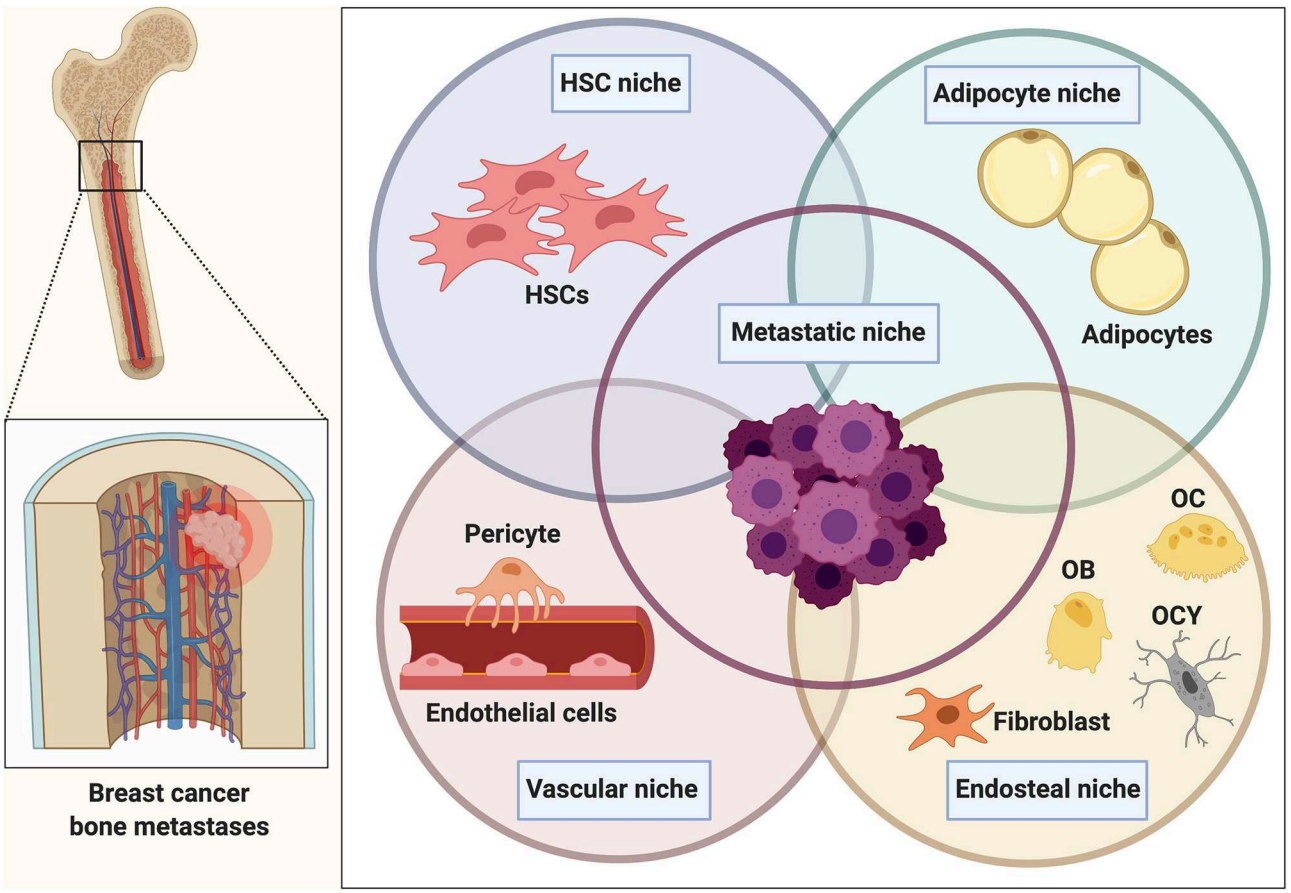

FIGURE 1 | The bone metastatic niche. Once homed to bone, tumor cells are exposed to a heterogeneous microenvironment that is comprised of various individual cellular entities. The complex interplay between osteoblasts and osteoclasts during bone remodeling in addition to the presence of various other bone marrow-derived populations makes the bone microenvironment a favorable and supportive environment (metastatic niche) for disseminated cancer cells. Within bone, the metastatic niche is thought to be comprised of a hematopoietic stem cell niche (HSCs), endosteal (osteoclasts (OC), osteoblasts (OB), osteocytes (OCY), fibroblasts), and vascular niche (endothelial cells, pericytes). Several findings also implicate a role of the bone marrow adipocyte niche in bone metastasis. The interaction and overlap between the niches remain to be determined and resulted in the generalized term of the "metastatic niche" that is thought to regulate homing, survival and dormancy of tumor cells.

In addition to the vascular niche and the endothelial niche, the adipocytes have been proposed as important players of the metastatic niche. Bone marrow adipocity increases during aging and thus, the potential role of the adipogenic niche becomes increasingly important in the elderly suffering from breast cancer. Indeed, in a model of human bone tissue, breast cancer cells were shown to migrate into the bone marrow adipose tissue and establish direct cellular interactions with the adipocytes (24). The recruitment was shown to be mediated by adipose-derived leptin and interleukin (IL)-1β, highlighting the role of cytokines and adipokines in breast cancer bone colonization.

OSTEOBLASTS IN TUMOR CELL HOMING, DISSEMINATION, AND DORMANCY

Osteoblasts are also suggested as potential mediators of breast cancer cell homing to bone (**Figure 2**). This arises from the observations that disseminated breast cancer cells are frequently found in bone areas that are rich in osteoblasts (29). This phenomenon could in part be mediated by the fact that osteoblasts express SDF-1 and RANKL, two cytokines that favor breast cancer cell dissemination and ultimately tumor growth through binding to their cognate receptors (CXCR4 and receptor activator of nuclear factor kappa-B (RANK), respectively) on the cancer cells (26, 30). Hypoxia-inducible factor (HIF)-signaling in osteoprogenitor cells for example has been shown to not only promote metastasis in the bones, but to also stimulate breast cancer cell dissemination to organs beyond the skeleton, such as for example the lung, partially through the production of SDF-1 (31). The hypothesis that tumor cells use the SDF-1/CXCR4 axis to hone to the osteoblastic niche in bone is supported by the finding that both, newly and established metastases are anchored in the bone marrow by SDF-1/CXCR4 interactions (26). Already in 2006, Phadke and colleagues reported that the majority of disseminated MDA-MB-435 breast cancer cells located in the primary spongiosa of the metaphysis of the distal femur, where metastatic growth ultimately proceeded (32). Furthermore, in an intracardiac model using BALB/c nude mice MDA-MB-231 breast cancer cells preferentially localized in the metaphysis, and especially close to trabecular bone surfaces that are rich in osteoblasts (29). Consistently, early metastases of a breast cancer cell line obtained from MMTV-PyMT mice were found adjacent to trabecular bone areas below the growth plate cartilage that was enriched in osteoprogenitor cells (OPN^{high}, $SDF\text{-}1^{high}$) (31). The metaphysis might provide a rich reservoir of growth factors, especially through the dense, interconnected vascular system (33). Although there might be a differential expression of adhesion molecules and growth factors in this area, studies have also shown that Runx2, Col1α, and Osx-positive osteoblasts are abundantly located around CD31-positive bone marrow vessels in the metaphysis (33).

Once in the bone, tumor cells can remain dormant for decades until the development of metastatic disease. Importantly, it still remains unknown what triggers the initiation from dormant DTCs or micrometastases into actively proliferating metastases. It has been suggested that osteoblasts, upon the presence of breast cancer cells, might produce soluble factors that act as chemoattractants, maintenance and/or growth factors for both, breast cancer cells and/or osteoclasts. Consequently, this would result in the activation of the vicious cycle of bone metastasis and osteolytic disease (34). Studies by Kinder and colleagues report that metastatic MDA-MB-231 breast cancer cells increase the production of inflammatory cytokines such as IL-6, monocyte chemoattractant protein-1 (MCP-1), and IL-8 in both, human hFOB 1.19, and murine M3T3-E1 osteoblasts (35). Similar results are documented by Bussard and colleagues showing an increased presence of osteoblast-derived cytokines including IL-6, IL-8, MCP-1, macrophage-inflammatory protein 2 (MIP-2), and vascular endothelial growth factor (VEGF) in the presence of MDA-MB-231 breast cancer cells in *ex vivo* cultures of tumor bearing bones from athymic mice, or even in the presence of conditioned medium *in vitro* (34). MCP-1 for example is known to be involved in osteoclastogenesis as well as in the attraction and infiltration of monocytes and macrophages during inflammation (36). In addition, MCP-1 has been shown not only to be expressed and secreted by breast cancer cells, but also to increase breast cancer cell invasiveness *in vitro* (37).

Studies by Wang and colleagues propose that the microenvironment of microscopic bone metastases in breast cancer is primarily composed of osteoblastic cells (7). The authors characterized the cellular composition of the bone niche in the presence of triple negative (Estrogen receptor (ER-), progesterone receptor (PR-) and HER/Neu -negative, MDA-MB-231) or estrogen-receptor positive (ER+, MCF-7) breast cancer metastases *in vivo*. They observed an increase of tartrate-resistant acid phosphatase (TRAP) -positive osteoclasts during the transition from indolent, non-proliferative micrometastases to the osteolytic cycle. In contrast, during the pre-osteolytic stage under 20% of niche cells surrounding the microscopic breast cancer bone metastases could be accounted to the osteoclastic lineage. Furthermore, the cathepsin K-positive osteoclasts were not in direct contact with the cancer cells. However, around 80% of the cells adjacent to the breast cancer micrometastases abundantly expressed ALP and around 50% of the cells were positive for Col1, both markers for cells of the osteoblastic lineage (7). Furthermore, compared to tumor-free bones there was an enrichment of ALP and ColI -positive cells in bones containing micrometastases, suggesting that osteoblasts facilitate breast cancer cell colonization in the bone environment. Importantly niche cells showed active features of osteogenesis including the expression of Runx2 and Osx, regulators of osteoblast differentiation, as well as active Wnt signaling. Breast cancer cell—osteoblast interaction was mediated via heterotypic adherens junctions using E-, and N-cadherins. Consequently, this interaction resulted in an enhanced mTOR activity in cancer cells and was associated with the transition from DTCs into overt metastases (7), suggesting a potential route of how osteoblasts could regulate breast cancer cell dormancy in the bone.

While Wang and colleagues propose that osteoblasts would rather initiate metastatic tumor growth in bone and/or facilitate escape from dormancy, recently published studies by Kolb and colleagues identified a subtype of osteoblasts—termed tumor educated osteoblasts (EOs)—that have a functional role in suppressing breast cancer growth (8). Upon contact with tumor cells a subpopulation of osteoblasts was educated by the cancer

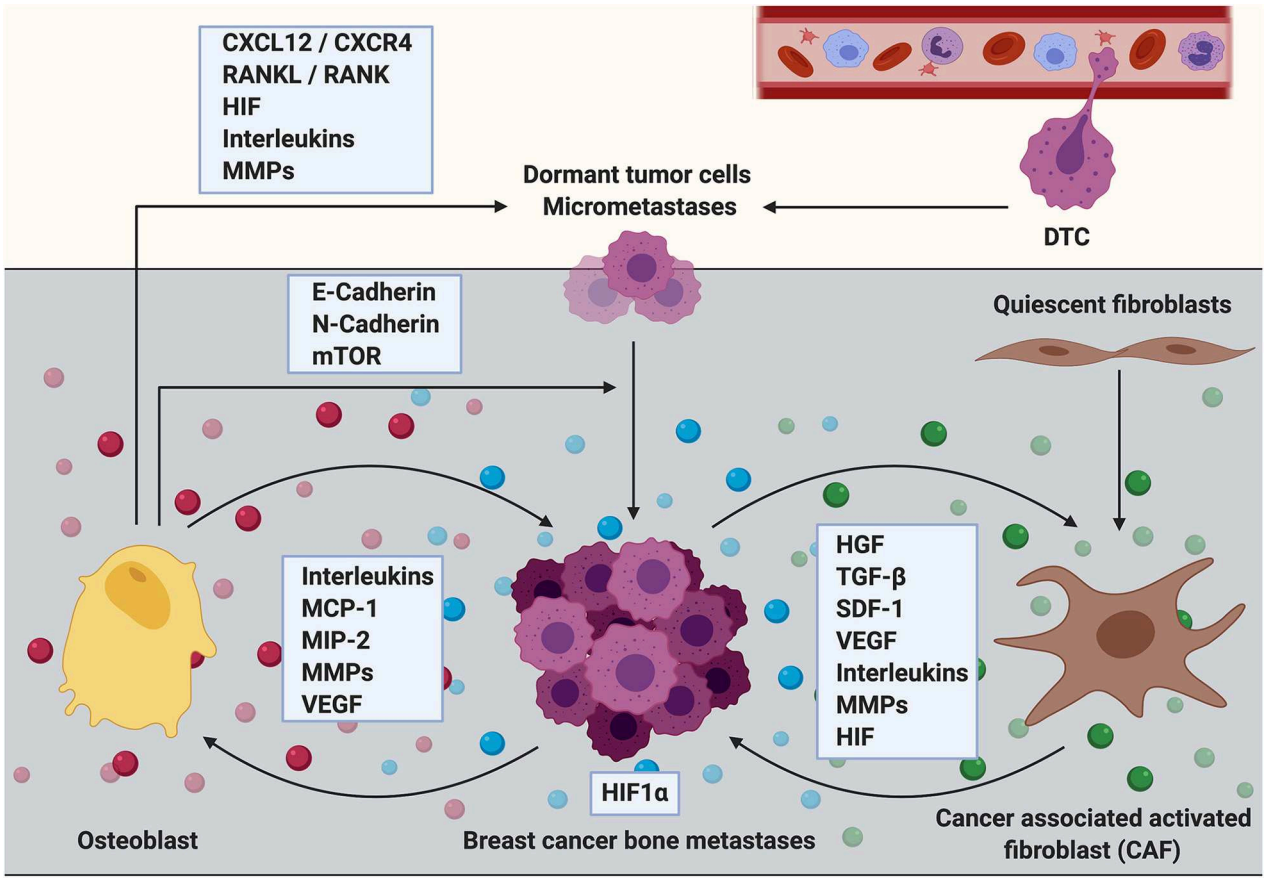

FIGURE 2 | The role of osteoblasts and cancer associated fibroblasts during the establishment and progression of breast cancer bone metastasis. Cells of mesenchymal origin including osteoblasts and cancer associated fibroblasts (CAFs) are increasingly recognized to contribute to the establishment and progression of breast cancer bone metastasis. Osteoblasts express cytokines including C-X-C motif chemokine ligand 12 (CXCL12, also referred to as stromal derived factor 1, SDF-1) and receptor activator of NF-κB ligand (RANKL) that promote breast cancer cell dissemination and metastatic growth through interaction with their corresponding receptors (CXCR4 and RANK, respectively) that are expressed by the cancer cells. Breast cancer micrometastases have also been shown to be surrounded by osteoblastic cells. The interaction between breast cancer cells and osteoblasts could partially be mediated via heterotypic adherens junctions using E-, and N-cadherins resulting in an enhanced mTOR activity in cancer cells and consequently in the transition from dormant tumor cells into overt metastases. Osteoblasts also express high levels of extracellular matrix remodeling proteins (MMPs) in addition to reduced presence of inflammatory cytokines such as interleukins (ILs) upon cancer cell stimulation. Thereby osteoblasts could regulate breast cancer cell dormancy in the bone microenvironment. In contrast, metastatic breast cancer cells increase the production of inflammatory cytokines such as IL-6 and IL-8, monocyte chemoattractant protein−1 (MCP-1), macrophage−inflammatory protein 2 (MIP-2) and vascular endothelial growth factor (VEGF) in osteoblasts, thereby promoting breast cancer cell invasiveness and metastasis progression. Although usually quiescent in normal tissue, fibroblasts acquire an activated phenotype during processes such as wound healing or inflammation. Activated fibroblasts in the tumor stroma are called cancer associated fibroblasts (CAFs). They produce growth factors that contribute to disease progression including hepatocyte growth factor (HGF), transforming growth factor beta (TGF-β), stromal derived factor 1 (SDF-1 or CXCL12), VEGF, IL-6, and other ILs in addition to MMPs. All of these factors promote primary tumor growth and it can be hypothesized that CAFs could similarly mediate the growth of breast cancer bone metastases. CAFs are known to induce extracellular matrix remodeling and alter the stiffness of tissues thereby facilitating tumor cell invasion, dissemination and/or metastasis. CAF-induced matrix remodeling and CAF invasion have been shown to be supported by hypoxia inducible factor−1 alpha (HIF1α). In turn, an increased expression of HIF1α might stimulate the tumor growth promoting function of CAFs.

cells into an osteopontin^{high} and αSMA^{low} phenotype *in vivo*. To further characterize the EOs, MC3T3-E1 cells were incubated *in vitro* with conditioned medium from MDA-MB-231 or MCF-7 breast cancer cells. Upon cancer cell stimulation EOs demonstrated lower abundance of the inflammatory cytokine IL-6 and increased expression of ECM remodeling proteins such as matrix metalloprotease 3 (MMP3) and Col1. Furthermore, conditioned medium from EOs retarded the proliferation of both, the metastatic MDA-MB-231 and the estrogen receptor positive MCF-7 breast cancer cell line *in vitro* as a reduced number of breast cancer cells entered the S-phase of the cell cycle. These studies suggest that distinct subpopulations of osteoblasts could contribute differently to tumor cell dormancy (8).

THE ROLE OF OSTEOBLASTS DURING BONE METASTASES PROGRESSION

Little focus has been put on investigating the interaction between osteoblasts and breast cancer cells during bone metastases

progression, mainly due to the fact that osteoblast numbers decrease during the advancement of osteolytic disease. By analyzing the distribution of metastatic MDA-MB-435 breast cancer cells in female athymic mice over a time period of 1 h to 6 weeks, Phadke and colleagues observed that breast cancer micrometastases (<10 cells) resided in great proximity to osteoblastic cells, whereas the number of osteoblasts decreased as tumor burden increased (32). Also studies by Brown and colleagues report that the presence of tumor cells modifies the osteoblast-osteoclast ratio in the bone microenvironment, and that these changes largely depend on whether there is direct contact between bone and tumor cells (38). In these studies, the effect of tumor cells on osteoblasts was most profound prior to the initiation of osteolytic disease. Compared to naïve mice, osteoblast number per mm trabecular bone surfaces was significantly increased in tumor bearing mice prior to the onset of metastatic bone disease, followed by a decrease in the osteoblast/osteoclast ratio once osteolytic lesions were apparent. Interestingly though, a more detailed analysis of osteoblasts adjacent and distant to the tumor cells revealed that the number of osteoblasts distant from the tumor cells was increased compared to those in direct contact with the tumor (38).

These data suggest a key role of osteoblasts during the early stages of breast cancer bone metastasis. Several *in vitro* studies support this hypothesis. For instance, osteoblast conditioned medium can act as a chemoattractant for breast cancer cells. A 12% increase in cell migration was observed when MDA-MB-231 breast cancer cells were allowed to migrate toward medium conditioned by osteoblasts compared to control medium using the transwell migration assay (34). Using the wound healing assay, pre-osteoblasts (ALP^{low}, OPN^{low}, $Runx2^{high}$, Osx^{high}, $CD166^{high}$) but not mature osteoblasts were shown to enhance the migration of MDA-MB-231 breast cancer cells (39). By using a vybrant cell adhesion kit the authors were also able to show that adhesion of MDA-MB-231 breast cancer cells to pre-osteoblastic cells was strongly increased when compared to undifferentiated cells or mature osteoblasts, suggesting that osteoblasts regulate early stages involved in metastatic breast cancer growth (39). Vice versa, data also suggest that breast cancer cells can stimulate the migration of mesenchymal cells, progenitors of osteoblasts (40). In contrast, a specific sub-type of osteoblasts [OPN^{high} and alpha-smooth muscle actin (αSMA^{low})] has been shown to retard breast cancer cell proliferation (8). In summary, these findings highlight that there is indeed an interaction between osteoblasts and breast cancer cells during the early stages of breast cancer bone metastases and that these communications could determine whether tumor cells undergo dormancy or whether they develop into overt metastases.

OSTEOBLASTS AS NOVEL TARGET TO TREAT BONE METASTASES—BONE ANABOLIC TREATMENT

Advancements have been made in limiting progression of breast cancer bone metastasis and novel therapeutic agents are emerging (41). However, once osteolytic lesions have been developed, the disease remains incurable and treatment is restricted to palliative care. This often includes the administration of the anti-resorptive bisphosphonate Zoledronic acid, or of the RANKL inhibitor Denosumab to reduce the cancer-induced bone destruction (42–44) (**Figure 3**). Further experimental approaches to target osteoclasts in metastatic bone disease include Cathepsin-K and c-Src inhibitors (45, 46). However, these agents are not able to restore the cancer-induced bone destruction. Therefore, augmenting osteoblast function has been proposed as a potential approach to restore bone integrity in the context of metastasis-induced osteolytic lesions (47).

Osteoporosis is a debilitating disease that leads to loss of bone mass and ultimately results in fragility fractures (48), similarly as in cancer-induced bone disease. To date, three bone anabolic drugs are available in the clinic for the treatment of severe osteoporosis. Two of the drugs are based on the activation of the PTH receptor by an intermittent administration of a recombinant fragment of PTH (Teriparatide; Forteo/Forsteo) or Parathyroid hormone related protein (PTHrP; Abaloparatide) (49). Recently, the effect of PTH on breast cancer bone metastasis was investigated in two studies. A short term (5 days, daily) administration of PTH prior to intracardial injection of MDA-MB-231 breast cancer cells was shown to have no effect on tumor cell homing or growth in the hind limbs of mice (50). However, tumor burden was increased in other skeletal sites suggesting that PTH-mediated alteration of the endosteal niche renders different skeletal sites to cancer cell colonization (50). In contrast, an anabolic (4 weeks, daily) treatment of mice with PTH was demonstrated to prevent skeletal metastases and preserve bone architecture in orthotopic and intratibial breast cancer models (51). Despite different experimental design, which is likely to explain the different results, both studies demonstrate that alteration of the bone microenvironment and osteoblast function by PTH affect breast cancer bone colonization. However, the use of Teriparatide is not approved for use in patients with a history of primary or metastatic bone cancer (52).

The third bone anabolic agent is an antibody against the Wnt signaling inhibitor Sclerostin (Scl-Ab; Romosozumab) that increases bone formation and bone mass by activating the Wnt pathway in osteoblasts (49, 53). In clinical trials, sclerostin antibody treatment of women with postmenopausal osteoporosis increased bone formation, while bone resorption was decreased, leading to an increase in bone mineral density and a reduction of the fracture rate at several sites, including the hip and spine (54). Similarly, the bone anabolic and anti-resorptive effect of Scl-Ab was recently demonstrated in a pre-clinical mouse model of bone metastases (55). Importantly, Scl-Ab treatment not only reduced metastatic breast cancer burden *in vivo* but also protected from cancer-induced bone and muscle loss and increased survival of cancer-bearing animals (55). In addition, further agents targeting osteoblast differentiation and function have been investigated for the treatment of bone metastasis in various cancers, with potential benefits also for breast cancer-induced bone disease (41). These include for example inhibitors of Dickkopf 1 (Dkk-1) and Activin-A

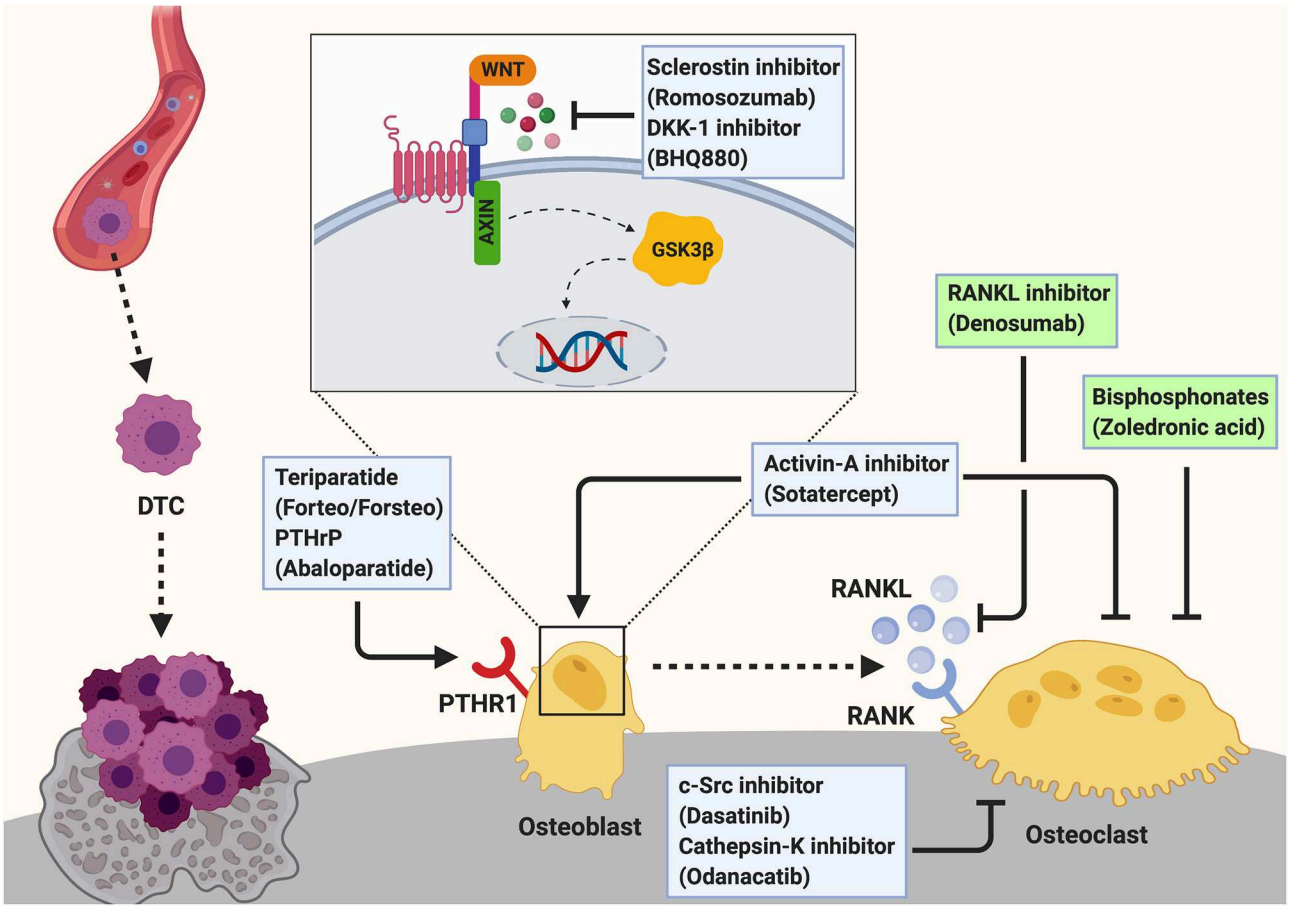

FIGURE 3 | Targeting the osteogenic niche to treat breast cancer bone metastasis. Breast cancer bone metastases remain incurable once osteolytic lesions have developed. Palliative treatment often includes the administration of osteoclast-targeted, anti-resorptive agents including bisphosphonates (e.g., Zoledronic acid) or the anti-RANKL antibody Denosumab to prevent the cancer-induced bone resorption. These two agents are the only approved treatments for cancer induced bone disease (indicated by green box). Additionally, c-Src (Dasatinib) and Cathepsin-K (Odanacatib) inhibitors are under investigation for the treatment of breast cancer bone metastasis. As these anti-resorptive agents are not able to restore the cancer-induced bone loss, augmenting osteoblast function by anabolic treatments has been proposed as a potential therapeutic approach and several agents are investigated experimentally and/or in clinical trials. Bone anabolic treatments including the administration of a recombinant fragment of PTH (Teriparatide; Forteo/Forsteo) or Parathyroid hormone related protein (PTHrP; Abaloparatide) are approved for the treatment of osteoporosis. However, these drugs cannot be prescribed for patients with bone metastases. Another bone anabolic agent Romosozumab, an antibody against the Wnt signaling inhibitor Sclerostin, increases bone formation and bone mass by activating the Wnt pathway in osteoblasts. Similarly, Dkk-1 inhibitors (e.g., BHQ880) allow active Wnt signaling in osteoblasts thereby increasing osteoblast activity. Inhibition of Activin-A signaling has been shown to prevent cancer-induced bone destruction. Additionally, Activin-A inhibitors (e.g., Sotatercept) have been shown to stimulate osteoblastogenesis while decreasing osteoclast activity to promote bone formation. Hence, they could potentially be a novel approach for the treatment of cancer induced bone disease.

(**Figure 3**). Similar to sclerostin, Dkk1 antagonizes Wnt signaling in osteoblasts. Consequently, inhibition of Dkk1 resulted in increased bone formation and reduced osteolysis in a mouse model of multiple myeloma, highlighting the potential benefit as an osteoanabolic agent (41, 56). Inhibition of Activin-A signaling with a soluble activin receptor type IIA fusion protein (ActRIIA.muFc) has been shown to stimulate osteoblastogenesis, promote bone formation and to inhibit bone metastasis and prevent bone destruction in a murine model of breast cancer bone metastasis (57). Although more investigation is needed, these studies suggest that targeting the endosteal niche by bone anabolic treatments could be a future approach to treat osteolytic bone metastases.

CANCER ASSOCIATED FIBROBLASTS IN BREAST CANCER (BONE) METASTASIS

As discussed in the previous sections, osteoblasts that originate from MSCs are increasingly recognized as therapeutic targets for breast cancer bone metastasis (8, 29). Another mesenchymal-derived, endosteal niche cell type with a potential to regulate the establishment and progression of bone metastasis includes fibroblasts. Although usually quiescent in normal tissue, fibroblasts acquire an activated phenotype during processes such as wound healing, tissue inflammation or fibrosis. Given the physiological role of fibroblasts, their involvement in tumor growth is apparent as cancers are considered as "wounds that

do not heal" (58). Cancer-associated fibroblasts (CAFs), activated fibroblasts that are associated with cancer, are one of the most abundant stromal cell types in breast cancer and are associated with poor prognosis (59) (**Figure 2**).

The contribution of CAFs in cancer progression has been extensively reviewed elsewhere (60, 61). Briefly CAFs produce growth factors that contribute to disease establishment (e.g., hepatocyte growth factor (HGF), TGF-β, SDF-1, VEGF, IL-6) in addition to MMPs. All of these factors are well-known to affect several hallmarks of cancer (60, 61). Whereas, the contribution of CAFs to primary tumor growth is intensely investigated and defined, the origin and role of CAFs in the metastatic environment, especially in breast cancer bone metastasis, remain poorly defined (62). Within the next chapters we discuss evidence that supports a role of CAFs during the progression and establishment of breast cancer bone metastasis.

ORIGIN AND CHARACTERIZATION OF CAFs IN THE TUMOR MICROENVIRONMENT

The origin of CAFs in the tumor microenvironment remains to be elucidated, but they might be derived from resident fibroblasts (63), actively recruited bone marrow-derived cells (64) or cells that undergo epithelial-mesenchymal transition (EMT) (65).

Due to the phenotypical and functional heterogeneity of CAFs there are no unique markers to identify them but commonly used ones include αSMA, fibroblast-specific protein1 (FSP1 or S100A4), fibroblast activation protein (FAP), platelet derived growth factor receptors (PDGFRα/β), vimentin, and tenascin C (66–68). Several *in vitro* studies demonstrate that MSCs can differentiate into αSMA -expressing myofibroblasts upon cancer cell stimulation (66, 69). For instance, studies by Mishra and colleagues show that human bone marrow-derived MSCs can acquire a CAF-like, myofibroblastic phenotype upon prolonged stimulation with conditioned medium from MDA-MB-231 breast cancer cells. Importantly, these cells expressed CAF markers including αSMA, SDF-1, vimentin, and FSP as determined by immunofluorescence staining. Gene expression analysis revealed that cancer-conditioned medium upregulated the expression of CAF-associated genes including SDF-1, platelet derived growth factor α (PDGFα) and MMP9, suggesting that exposure to cancer cells induces hMSC differentiation into a CAF-resembling state (66).

THE ROLE OF CAFs DURING THE ESTABLISHMENT AND PROGRESSION OF BREAST CANCER BONE METASTASIS

CAFs are thought contribute to both, primary and secondary breast cancer through regulating processes such as breast cancer cell proliferation and stemness as well as ECM remodeling, production and stiffness (67). Furthermore, CAFs are involved in regulating cancer cell migration, invasion and distant metastasis (58, 60, 70). Certain survival pathways including the c-Src pathway are suggested to be detrimental for metastatic latency. Indeed, using a gene expression profiling Zhang and colleagues revealed a strong correlation between c-Src activity and bone metastasis [a Src response signature (SRS) (71). Further studies linked the SRS and the CAF-content of primary breast tumors to the likelihood of these tumors to relapse in bone (72). In these experiments the authors demonstrate that triple negative breast cancers with a high SRS (SRS+) and therefore a high preference to metastasize to bone, had increased expression of CXCL12/14 and IGF-1/2 when compared to SRS- tumors. Interestingly, CAFs were identified as the source of these cytokines rather than the tumor cells themselves. Consequently, the authors suggest that a high prevalence of mesenchymal cells including CAFs in the stroma of triple negative breast tumors would select for certain clones. These include in particular clones that grow well under the presence of CAF-derived cytokines including CXCL12 or IGF-1. This in turn would lead to a predisposition of disseminated tumor cells to colonize the bone marrow which has a higher abundance of stromal -derived CXCL12 and IGF-1 compared to other metastatic sites such as lung, liver and brain (72). Additionally, it has been demonstrated that fibroblasts isolated from different sites of breast cancer growth, including the breast, lung and bone, enhance the invasiveness of ER+ breast cancer cells in an IL-6 dependent way (73). Furthermore, CAF-induced ECM remodeling and altered tissue stiffness might contribute to tumor cell invasiveness, dissemination and/or metastasis. Studies by Madsen and colleagues demonstrated that hypoxia reduced periostin and αSMA expression in CAFs, two common markers that indicate CAF activation (67). HIFs mediate response to hypoxia and in these studies HIF-1α supported CAF-induced matrix remodeling and invasion. Prolyl hydroxylase domain-containing proteins (PHDs) are enzymes that target the alpha subunits of HIF complexes for degradation under normoxic conditions. Interestingly loss or inhibition of PHD2 suppressed CAF induced matrix remodeling and invasion *in vitro*. In a 4T1 mouse model, inhibition of PHDs reduced stiffness of primary 4T1 tumors as well as the development of spontaneous metastasis to lung and liver (67). The authors suggest that targeting PHD2 in CAF-enriched tumors, including breast cancer, may have beneficial effects on metastasis development. However, metastasis to bone was not assessed in this study. Besides being a highly vascularized tissue, the bone microenvironment is hypoxic and regional oxygen tensions vary depending on the level of cellularity, oxygen consumption and supply of oxygenated blood. Hypoxia and activation of HIF1α as well as HIF2α is known to contribute to tumor progression and metastasis in various organs including breast cancer (74), but besides the studies by Devignes and colleagues (31) little is known about the impact of hypoxia in breast cancer bone metastasis. Studies by Hiraga and colleagues demonstrated that increased HIF1 α expression in MDA-MB-231 breast cancer cells enhanced the colonization after intracardiac inoculation (75). These findings provide room to speculate a role of CAFs in promoting and/or regulating breast cancer bone metastasis (67).

CONCLUSION/PERSPECTIVE

Over the last decade significant progress has been made in understanding metastatic breast cancer growth in bone. However, the disease remains incurable once tumor cells are actively proliferating in bone. Many aspects, in particular the initial stages of bone metastasis, need to be investigated further in order to prevent disease establishment.

Research has recently focused on deciphering the early events of metastatic tumor growth in bone, including the entry and exit from dormancy or the transition from micro-metastases to overt metastases (7, 24, 27, 28, 76). Substantial evidence exists that cancer cells interact with cells of the bone microenvironment to render physiological processes and/or cell to cell communications in order to promote tumor cell maintenance, survival and proliferation in bone (3, 7, 34, 77–79).

One important component of the (bone) tumor microenvironment includes mesenchymal-derived cells including osteoblasts and fibroblasts or so called "endosteal niche cells." Endosteal niche cells and in particular osteoblasts are increasingly appreciated as important components of the metastatic niche (8, 34, 35, 38, 39, 80, 81). However, their role in supporting tumor cell homing, dormancy and disease progression remains poorly defined. Unlike osteoclasts, the contribution of osteoblasts to breast cancer bone metastasis remained under-investigated over the last years. Nevertheless, recently published studies highlight their potential as novel cellular targets to prevent and/or treat breast cancer bone metastasis (7, 8, 29, 34, 38, 39, 75). In addition, the therapeutic importance of osteoblasts has been acknowledged with novel therapeutics including bone anabolic agents such as PTH and anti-sclerostin antibody (51, 55). However, a detailed characterization of how bone anabolic agents modify the composition and/or location of the endosteal niche as well as potential consequences on tumor cell colonization and metastatic outgrowth remains to be performed. In addition, further research is needed to investigate whether stimulating osteoblast activity would result in the repair of osteolytic bone lesions. This also raises the question whether a combination of anti-osteolytic and bone anabolic therapy would be beneficial for the treatment of breast cancer bone metastasis.

Another mesenchymal-derived cell type in the bone microenvironment includes fibroblasts, which transform into CAFs upon the presence of disseminated tumor cells. CAFs are known to create a tumor permissive environment by influencing nearly all hallmarks of breast cancer (60, 61, 82). The role of CAFs in promoting tumor growth is evident, they release growth factors, stimulate angiogenesis, proliferation, migration as well as ECM remodeling. However, these findings are primarily derived from research that has been limited to the primary tumor. In contrast, little is known about their role in the metastatic environment. Especially the contribution of CAFs to the establishment and progression of breast cancer bone metastasis is poorly defined (64–67). Another open question remains the origin of CAFs in the metastatic (bone) environment. Identifying the origin of CAFs would provide targets to suppress their tumor growth-supporting function.

In summary, a deeper understanding of the interaction between the endosteal cell compartment and disseminated breast cancer cells will be needed to develop more successful treatment for breast cancer bone metastases. Research techniques to investigate cell-cell interactions, especially *in vitro*, have significantly improved over the last years. Nevertheless, our ability to track and visualize these interactions *in vivo* remains limited. This highlights the need to improve our model systems as well as imaging techniques to increase our knowledge about the interaction between tumor cells and cells of the microenvironment. Consequently, this will aid to elucidate the mechanisms of how osteogenic cells suppress or promote metastatic growth in bone and would provide novel therapeutic targets that could be used to maintain disseminated tumor cells in a dormant state or to completely prevent dissemination/colonization in the bone.

AUTHOR CONTRIBUTIONS

MTH and HT reviewed the literature and wrote the manuscript. DS reviewed the literature and prepared the figures.

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REFERENCES

1. Weilbaecher KN, Guise TA, McCauley LK. Cancer to bone: a fatal attraction. *Nat Rev Cancer*. (2011) 11:411–25. doi: 10.1038/nrc3055
2. Paget S. The distribution of secondary growths in cancer of the breast. 1889. *Cancer Metastasis Rev*. (1989) 8:98–101.
3. Croucher PI, McDonald MM, Martin TJ. Bone metastasis: the importance of the neighbourhood. *Nat Rev Cancer*. (2016) 16:373–86. doi: 10.1038/nrc.2016.44
4. Mundy GR. Mechanisms of bone metastasis. *Cancer*. (1997) 80(Suppl. 8):1546–56. doi: 10.1002/(SICI)1097-0142(19971015)80:8+<1546::AID-CNCR4>3.0.CO;2-I
5. Clines GA, Guise TA. Molecular mechanisms and treatment of bone metastasis. *Expert Rev Mol Med*. (2008) 10:e7. doi: 10.1017/S1462399408000616
6. Haider M-T, Taipaleenmäki H. Targeting the metastatic bone microenvironment by MicroRNAs. *Front Endocrinol (Lausanne)*. (2018) 9:202. doi: 10.3389/fendo.2018.00202
7. Wang H, Yu C, Gao X, Welte T, Muscarella AM, Tian L, et al. The osteogenic niche promotes early-stage bone colonization of disseminated breast cancer cells. *Cancer Cell*. (2015) 27:193–210. doi: 10.1016/j.ccell.2014.11.017
8. Kolb AD, Shupp AB, Mukhopadhyay D, Marini FC, Bussard KM. Osteoblasts are "educated" by crosstalk with metastatic breast cancer cells

in the bone tumor microenvironment. *Breast Cancer Res.* (2019) 21:31. doi: 10.1186/s13058-019-1117-0
9. Méndez-Ferrer S, Michurina TV, Ferraro F, Mazloom AR, MacArthur BD, Lira SA, et al. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature*. (2010) 466:829–34. doi: 10.1038/nature09262
10. Zaidi M. Skeletal remodeling in health and disease. *Nat Med.* (2007) 13:791–801. doi: 10.1038/nm1593
11. Harada S, Rodan GA. Control of osteoblast function and regulation of bone mass. *Nature*. (2003) 423:349–55. doi: 10.1038/nature01660
12. Baron R, Kneissel M. WNT signaling in bone homeostasis and disease: from human mutations to treatments. *Nat Med.* (2013) 19:179–92. doi: 10.1038/nm.3074
13. Taipaleenmäki H, Abdallah BM, AlDahmash A, Säämänen AM, Kassem M. Wnt signalling mediates the cross-talk between bone marrow derived pre-adipocytic and pre-osteoblastic cell populations. *Exp Cell Res.* (2011) 317:745–56. doi: 10.1016/j.yexcr.2010.12.015
14. Teitelbaum SL. Osteoclasts: what do they do and how do they do it? *Am J Pathol.* (2007) 170:427–35. doi: 10.2353/ajpath.2007.060834
15. Sims NA, Martin TJ. Coupling signals between the osteoclast and osteoblast: how are messages transmitted between these temporary visitors to the bone surface? *Front Endocrinol (Lausanne).* (2015) 6:41. doi: 10.3389/fendo.2015.00041
16. Butler JM, Kobayashi H, Rafii S. Instructive role of the vascular niche in promoting tumour growth and tissue repair by angiocrine factors. *Nat Rev Cancer*. (2010) 10:138–46. doi: 10.1038/nrc2791
17. Park D, Sykes DB, Scadden DT. The hematopoietic stem cell niche. *Front Biosci.* (2012) 17:30. doi: 10.2741/3913
18. Stegen S, Carmeliet G. The skeletal vascular system – Breathing life into bone tissue. *Bone*. (2018) 115:50–8. doi: 10.1016/j.bone.2017.08.022
19. Kaplan RN, Riba RD, Zacharoulis S, Bramley AH, Vincent L, Costa C, et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature*. (2005) 438:820–7. doi: 10.1038/nature04186
20. Malanchi I, Santamaria-Martínez A, Susanto E, Peng H, Lehr H-A, Delaloye J-F, et al. Interactions between cancer stem cells and their niche govern metastatic colonization. *Nature*. (2012) 481:85–9. doi: 10.1038/nature10694
21. Erler JT, Bennewith KL, Nicolau M, Dornhöfer N, Kong C, Le Q-T, et al. Lysyl oxidase is essential for hypoxia-induced metastasis. *Nature*. (2006) 440:1222–6. doi: 10.1038/nature04695
22. Cox TR, Rumney RMH, Schoof EM, Perryman L, Høye AM, Agrawal A, et al. The hypoxic cancer secretome induces pre-metastatic bone lesions through lysyl oxidase. *Nature*. (2015) 522:106–10. doi: 10.1038/nature14492
23. Hoshino A, Costa-Silva B, Shen T-L, Rodrigues G, Hashimoto A, Tesic Mark M, et al. Tumour exosome integrins determine organotropic metastasis. *Nature*. (2015) 527:329–35. doi: 10.1038/nature15756
24. Templeton ZS, Lie W-R, Wang W, Rosenberg-Hasson Y, Alluri RV, Tamaresis JS, et al. Breast cancer cell colonization of the human bone marrow adipose tissue niche. *Neoplasia*. (2015) 17:849–61. doi: 10.1016/j.neo.2015.11.005
25. Malladi S, Macalinao DG, Jin X, He L, Basnet H, Zou Y, et al. Metastatic Latency and Immune Evasion through Autocrine Inhibition of WNT. *Cell*. (2016) 165:45–60. doi: 10.1016/j.cell.2016.02.025
26. Price TT, Burness ML, Sivan A, Warner MJ, Cheng R, Lee CH, et al. Dormant breast cancer micrometastases reside in specific bone marrow niches that regulate their transit to and from bone. *Sci Transl Med*. (2016) 8:340ra73-340ra73. doi: 10.1126/scitranslmed.aad4059
27. Ghajar CM, Peinado H, Mori H, Matei IR, Evason KJ, Brazier H, et al. The perivascular niche regulates breast tumour dormancy. *Nat Cell Biol.* (2013) 15:807–17. doi: 10.1038/ncb2767
28. Capulli M, Hristova D, Valbret Z, Carys K, Arjan R, Maurizi A, et al. Notch2 pathway mediates breast cancer cellular dormancy and mobilisation in bone and contributes to haematopoietic stem cell mimicry. *Br J Cancer*. (2019) 121:157–71. doi: 10.1038/s41416-019-0501-y
29. Haider M-T, Holen I, Dear TN, Hunter K, Brown HK. Modifying the osteoblastic niche with zoledronic acid *in vivo*—Potential implications for breast cancer bone metastasis. *Bone*. (2014) 66:240–50. doi: 10.1016/j.bone.2014.06.023
30. Sun Y, Mao X, Fan C, Liu C, Guo A, Guan S, et al. CXCL12-CXCR4 axis promotes the natural selection of breast cancer cell metastasis. *Tumour Biol.* (2014) 35:7765–73. doi: 10.1007/s13277-014-1816-1
31. Devignes C-S, Aslan Y, Brenot A, Devillers A, Schepers K, Fabre S, et al. HIF signaling in osteoblast-lineage cells promotes systemic breast cancer growth and metastasis in mice. *Proc Natl Acad Sci.* (2018) 115:E992–E1001. doi: 10.1073/pnas.1718009115
32. Phadke PA, Mercer RR, Harms JF, Jia Y, Frost AR, Jewell JL, et al. Kinetics of metastatic breast cancer cell trafficking in bone. *Clin Cancer Res.* (2006) 12:1431–40. doi: 10.1158/1078-0432.CCR-05-1806
33. Kusumbe AP, Ramasamy SK, Adams RH. Coupling of angiogenesis and osteogenesis by a specific vessel subtype in bone. *Nature.* (2014) 507:323–8. doi: 10.1038/nature13145
34. Bussard KM, Venzon DJ, Mastro AM. Osteoblasts are a major source of inflammatory cytokines in the tumor microenvironment of bone metastatic breast cancer. *J Cell Biochem*. (2010) 111:1138–48. doi: 10.1002/jcb.22799
35. Kinder M, Chislock E, Bussard KM, Shuman L, Mastro AM. Metastatic breast cancer induces an osteoblast inflammatory response. *Exp Cell Res.* (2008) 314:173–83. doi: 10.1016/j.yexcr.2007.09.021
36. Miyamoto K, Ninomiya K, Sonoda KH, Miyauchi Y, Hoshi H, Iwasaki R, et al. MCP-1 expressed by osteoclasts stimulates osteoclastogenesis in an autocrine/paracrine manner. *Biochem Biophys Res Commun*. (2009) 383:373–7. doi: 10.1016/j.bbrc.2009.04.020
37. Dutta P, Sarkissyan M, Paico K, Wu Y, Vadgama JV. MCP-1 is overexpressed in triple-negative breast cancers and drives cancer invasiveness and metastasis. *Breast Cancer Res Treat*. (2018) 170:477–86. doi: 10.1007/s10549-018-4760-8
38. Brown HK, Ottewell PD, Evans CA, Holen I. Location matters: osteoblast and osteoclast distribution is modified by the presence and proximity to breast cancer cells *in vivo*. *Clin Exp Metastasis*. (2012) 29:927–38. doi: 10.1007/s10585-012-9481-5
39. Vallet S, Bashari MH, Fan FJ, Malvestiti S, Schneeweiss A, Wuchter P, et al. Pre-osteoblasts stimulate migration of breast cancer cells via the HGF/MET pathway. *PLoS ONE*. (2016) 11:e0150507. doi: 10.1371/journal.pone.0150507
40. Menon LG, Picinich S, Koneru R, Gao H, Lin SY, Koneru M, et al. Differential gene expression associated with migration of mesenchymal stem cells to conditioned medium from tumor cells or bone marrow cells. *Stem Cells*. (2007) 25:520–8. doi: 10.1634/stemcells.2006-0257
41. D'Oronzo S, Coleman R, Brown J, Silvestris F. Metastatic bone disease: Pathogenesis and therapeutic options: up-date on bone metastasis management. *J bone Oncol.* (2019) 15:004–4. doi: 10.1016/j.jbo.2018.10.004
42. Clezardin P, Teti A. Bone metastasis: pathogenesis and therapeutic implications. *Clin Exp Metastasis*. (2007) 24:599–608. doi: 10.1007/s10585-007-9112-8
43. Clézardin P. Mechanisms of action of bisphosphonates in oncology: a scientific concept evolving from antiresorptive to anticancer activities. *Bonekey Rep.* (2013) 2:267. doi: 10.1038/bonekey.2013.1
44. Coleman R. Bone targeted treatments in cancer - The story so far. *J bone Oncol.* (2016) 5:90–2. doi: 10.1016/j.jbo.2016.03.002
45. Rucci N, Recchia I, Angelucci A, Alamanou M, Del Fattore A, Fortunati D, et al. Inhibition of protein kinase c-Src reduces the incidence of breast cancer metastases and increases survival in mice: implications for therapy. *J Pharmacol Exp Ther*. (2006) 318:161–72. doi: 10.1124/jpet.106.102004
46. Le Gall C, Bellahcène A, Bonnelye E, Gasser JA, Castronovo V, Green J, et al. A cathepsin K inhibitor reduces breast cancer induced osteolysis and skeletal tumor burden. *Cancer Res.* (2007) 67:9894–902. doi: 10.1158/0008-5472.CAN-06-3940
47. Suvannasankha A, Chirgwin JM. Role of bone-anabolic agents in the treatment of breast cancer bone metastases. *Breast Cancer Res.* (2014) 16:484. doi: 10.1186/s13058-014-0484-9
48. Compston JE, McClung MR, Leslie WD. Osteoporosis. *Lancet*. (2019) 393:364–76. doi: 10.1016/S0140-6736(18)32112-3
49. Sølling ASK, Harsløf T, Langdahl B. Current status of bone-forming therapies for the management of osteoporosis. *Drugs Aging.* (2019) 36:625–38. doi: 10.1007/s40266-019-00675-8
50. Brown H, Allocca G, Ottewell P, Wang N, Brown N, Croucher P, et al. Parathyroid Hormone (PTH) increases skeletal tumour growth and alters tumour distribution in an *in vivo* model of breast cancer. *Int J Mol Sci.* (2018) 19:2920. doi: 10.3390/ijms19102920
51. Swami S, Johnson J, Bettinson LA, Kimura T, Zhu H, Albertelli MA, et al. Prevention of breast cancer skeletal metastases with parathyroid hormone. *JCI Insight.* (2017) 2:90874. doi: 10.1172/jci.insight.90874

52. Grey A. Teriparatide for bone loss in the jaw. *N Engl J Med.* (2010) 363:2458–9. doi: 10.1056/NEJMe1010459
53. Cosman F. The evolving role of anabolic therapy in the treatment of osteoporosis. *Curr Opin Rheumatol.* (2019) 31:376–80. doi: 10.1097/BOR.0000000000000616
54. Cosman F, Crittenden DB, Adachi JD, Binkley N, Czerwinski E, Ferrari S, et al. Romosozumab treatment in postmenopausal women with osteoporosis. *N Engl J Med.* (2016) 375:1532–43. doi: 10.1056/NEJMoa1607948
55. Hesse E, Schröder S, Brandt D, Pamperin J, Saito H, Taipaleenmäki H. Sclerostin inhibition alleviates breast cancer–induced bone metastases and muscle weakness. *JCI Insight.* (2019) 4. doi: 10.1172/jci.insight.125543
56. Eda H, Santo L, Wein MN, Hu DZ, Cirstea DD, Nemani N, et al. Regulation of sclerostin expression in multiple myeloma by Dkk-1: a potential therapeutic strategy for myeloma bone disease. *J Bone Miner Res.* (2016) 31:1225–34. doi: 10.1002/jbmr.2789
57. Chantry AD, Heath D, Mulivor AW, Pearsall S, Baud'huin M, Coulton L, et al. Inhibiting activin-A signaling stimulates bone formation and prevents cancer-induced bone destruction *in vivo*. *J Bone Miner Resv*. (2010) 25:2633–46. doi: 10.1002/jbmr.142
58. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med.* (1986) 315:1650–9. doi: 10.1056/NEJM198612253152606
59. Zhou J, Wang XH, Zhao YX, Chen C, Xu XY, Sun Q, et al. Cancer-associated fibroblasts correlate with tumor-associated macrophages infiltration and lymphatic metastasis in triple negative breast cancer patients. *J Cancer.* (2018) 9:4635–41. doi: 10.7150/jca.28583
60. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell.* (2000) 100:57–70. doi: 10.1016/S0092-8674(00)81683-9
61. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* (2011) 144:646–74. doi: 10.1016/j.cell.2011.02.013
62. Graham N, Qian BZ. Mesenchymal stromal cells: emerging roles in bone metastasis. *Int J Mol Sci.* (2018) 19:1121. doi: 10.3390/ijms19041121
63. Yin C, Evason KJ, Asahina K, Stainier DY. Hepatic stellate cells in liver development, regeneration, and cancer. *J Clin Invest.* (2013) 123:1902–10. doi: 10.1172/JCI66369
64. Raz Y, Cohen N, Shani O, Bell RE, Novitskiy SV, Abramovitz L, et al. Bone marrow–derived fibroblasts are a functionally distinct stromal cell population in breast cancer. *J Exp Med.* (2018) 215:3075–93. doi: 10.1084/jem.20180818
65. Bartoschek M, Oskolkov N, Bocci M, Lövrot J, Larsson C, Sommarin M, et al. Spatially and functionally distinct subclasses of breast cancer-associated fibroblasts revealed by single cell RNA sequencing. *Nat Commun.* (2018) 9:5150. doi: 10.1038/s41467-018-07582-3
66. Mishra PJ, Mishra PJ, Humeniuk R, Medina DJ, Alexe G, Mesirov JP, et al. Carcinoma-associated fibroblast-like differentiation of human mesenchymal stem cells. *Cancer Res.* (2008) 68:4331–9. doi: 10.1158/0008-5472.CAN-08-0943
67. Madsen CD, Pedersen JT, Venning FA, Singh LB, Moeendarbary E, Charras G, et al. Hypoxia and loss of PHD2 inactivate stromal fibroblasts to decrease tumour stiffness and metastasis. *EMBO Rep.* (2015) 16:1394–408. doi: 10.15252/embr.201540107
68. Houthuijzen JM, Jonkers J. Cancer-associated fibroblasts as key regulators of the breast cancer tumor microenvironment. *Cancer Metastasis Rev.* (2018) 37:577–97. doi: 10.1007/s10555-018-9768-3
69. Shangguan L, Ti X, Krause U, Hai B, Zhao Y, Yang Z, et al. Inhibition of TGF-β/Smad signaling by BAMBI blocks differentiation of human mesenchymal stem cells to carcinoma-associated fibroblasts and abolishes their protumor effects. *Stem Cells.* (2012) 30:2810–9. doi: 10.1002/stem.1251
70. O'Connell JT, Sugimoto H, Cooke VG, MacDonald BA, Mehta AI, LeBleu VS, et al. VEGF-A and Tenascin-C produced by S100A4+ stromal cells are important for metastatic colonization. *Proc Natl Acad Sci USA.* (2011) 108:16002–7. doi: 10.1073/pnas.1109493108
71. Zhang XH-F, Wang Q, Gerald W, Hudis CA, Norton L, Smid M, et al. Latent bone metastasis in breast cancer tied to Src-dependent survival signals. *Cancer Cell.* (2009) 16:67–78. doi: 10.1016/j.ccr.2009.05.017
72. Zhang XH-F, Jin X, Malladi S, Zou Y, Wen YH, Brogi E, et al. Selection of bone metastasis seeds by mesenchymal signals in the primary tumor stroma. *Cell.* (2013) 154:1060–73. doi: 10.1016/j.cell.2013.07.036
73. Studebaker AW, Storci G, Werbeck JL, Sansone P, Sasser AK, Tavolari S, et al. Fibroblasts isolated from common sites of breast cancer metastasis enhance cancer cell growth rates and invasiveness in an interleukin-6-dependent manner. *Cancer Res.* (2008) 68:9087–95. doi: 10.1158/0008-5472.CAN-08-0400
74. Chaturvedi P, Gilkes DM, Wong CCL, Kshitiz Luo W, Zhang H, et al. Hypoxia-inducible factor-dependent breast cancer-mesenchymal stem cell bidirectional signaling promotes metastasis. *J Clin Invest*. (2013) 123:189–205. doi: 10.1172/JCI69244
75. Hiraga T, Kizaka-Kondoh S, Hirota K, Hiraoka M, Yoneda T. Hypoxia and hypoxia-inducible factor-1 expression enhance osteolytic bone metastases of breast cancer. *Cancer Res*. (2007) 67:4157–63. doi: 10.1158/0008-5472.CAN-06-2355
76. Allocca G, Hughes R, Wang N, Brown HK, Ottewell PD, Brown NJ, et al. The bone metastasis niche in breast cancer-potential overlap with the haematopoietic stem cell niche *in vivo*. *J Bone Oncol*. (2019) 17:100244. doi: 10.1016/j.jbo.2019.100244
77. Allinen M, Beroukhim R, Cai L, Brennan C, Lahti-Domenici J, Huang H, et al. Molecular characterization of the tumor microenvironment in breast cancer. *Cancer Cell.* (2004) 6:17–32. doi: 10.1016/j.ccr.2004.06.010
78. Balkwill FR, Capasso M, Hagemann T. The tumor microenvironment at a glance. *J Cell Sci.* (2012) 125:5591–6. doi: 10.1242/jcs.116392
79. Coleman RE, Gregory W, Marshall H, Wilson C, Holen I. The metastatic microenvironment of breast cancer: clinical implications. *Breast.* (2013) 22:S50–6. doi: 10.1016/j.breast.2013.07.010
80. Thomas RJ, Guise TA, Yin JJ, Elliott J, Horwood NJ, Martin TJ, et al. Breast cancer cells interact with osteoblasts to support osteoclast formation. *Endocrinology.* (1999) 140:4451–8. doi: 10.1210/endo.140.10.7037
81. Adams CS, Shapiro IM. The fate of the terminally differentiated chondrocyte: evidence for microenvironmental regulation of chondrocyte apoptosis. *Crit Rev Oral Biol Med.* (2002) 13:465–73. doi: 10.1177/154411130201300604
82. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell.* (2012) 21:309–22. doi: 10.1016/j.ccr.2012.02.022

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REVIEW
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Current and Emerging Biomarkers Predicting Bone Metastasis Development

Michele Iuliani, Sonia Simonetti, Giulia Ribelli, Andrea Napolitano, Francesco Pantano, Bruno Vincenzi, Giuseppe Tonini and Daniele Santini*

Medical Oncology, Bio-Medico University of Rome, Rome, Italy

Bone is one of the preferential sites of distant metastases from malignant tumors, with the highest prevalence observed in breast and prostate cancers. Patients with bone metastases (BMs) may experience skeletal-related events, such as severe bone pain, pathological fractures, spinal cord compression, and hypercalcemia, with negative effects on the quality of life. In the last decades, a deeper understanding of the molecular mechanisms underlying the BM onset has been gained, leading to the development of bone-targeting agents. So far, most of the research has been focused on the pathophysiology and treatment of BM, with only relatively few studies investigating potential predictors of risk for BM development. The ability to select such "high-risk" patients could allow early identification of those most likely to benefit from interventions to prevent or delay BM. This review summarizes several evidences for the potential use of specific biomarkers able to predict early the BM development.

Keywords: bone metastases, CTCs, DTCs, ctDNA, miRNAs, bone turnover markers

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****Correspondence:***
Sonia Simonetti
s.simonetti@unicampus.it

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INTRODUCTION

Bone is a common site for tumor metastasis, particularly for breast, prostate, kidney, and lung cancers (1). Osteotropism is defined as the stepwise process whereby tumor cells acquire specific molecular characteristics that allow them to detach from the primary tumor and spread into the bloodstream and home within the bone niche. The highly vascular nature of the bone marrow, as well as the presence of pro-angiogenic cytokines and growth factors, contribute to the establishment of a favorable soil for cancer cells seeding and surviving in premetastatic sites. Once in the bone marrow, cancer cells (known as disseminated tumor cells, DTCs) may remain dormant or lead to the development of overt BM, even after prolonged periods of latency (2–5). The presence of DTCs in the bone marrow is correlated with an increased risk of disease recurrence and poor prognosis in early breast cancer (BCa) patients (6–8). Based on these evidences, bone-targeted agents' efficacy has been tested in adjuvant setting (9). In this regard, prospective randomized controlled trials have been designed showing conflicting results (9–14). In particular, the use of adjuvant bisphosphonates was associated to a reduction in the

incidence of BM, but benefits on overall survival were restricted to specific patient subgroups (10–14). Similar conflicting results were reported with adjuvant denosumab, a human monoclonal antibody that inhibits the receptor activator of nuclear factor κB ligand (RANKL). In the ABCSG-18 trial, adjuvant treatment with denosumab improved disease-free survival in patients with hormone receptor-positive BCa (15), whereas in the D-CARE trial, denosumab did not significantly increase BM-free survival in women with stage II or III BCa (16).

The identification at an early stage of the disease of patients at high risk for developing BM could consequently increase the impact by a bone-specific adjuvant treatment. Here, we report preclinical and clinical evidences on promising circulating and tissue biomarkers that could be useful for the prediction or early diagnosis of BM, as summarized in **Figure 1**.

EXPRESSION PROFILE IN PRIMARY TUMOR AS BIOMARKER FOR PREDICTING BONE METASTASES

Several authors reported that protein or gene expression profiles of the primary tumor might predict later BM development (**Table 1**). Westbrook et al. reported that the composite expression of the two proteins macrophage-capping protein (CAPG) and GIPC PDZ domain-containing protein (GIPC1) in primary BCa tissues of patients enrolled in the phase III AZURE trial strongly predicted skeletal disease-free survival (DFS) and overall survival (OS) (17). Interestingly, adjuvant zoledronate treatment significantly reduced distant bone recurrence only in patients with high expression of both proteins (17). These data suggest that CAPG and GIPC1 expression in primary BCa tissue might be both prognostic and predictive of efficacy with adjuvant zoledronate treatment. Xiao-Qing Li et al. identified integrin beta-like 1 (ITGBL1) as a candidate biomarker predicting BM development. Indeed, ITGBL1 was coexpressed with genes related to osteomimicry in primary BCa tissues and correlated with BM occurrence (18).

Moreover, gene expression and proteomics analysis on BCa cells more prone to cause BM in xenograft murine models might also help in the identification of relevant biomarkers. For example, interleukin (IL)-1β was found to be upregulated in a bone-seeking model of BCa cells, and further investigation on 150 primary BCa core biopsies showed a significant correlation between its expression and BM onset (19). Importantly, Holen et al. demonstrated the efficacy of IL-1β inhibitors in preventing skeletal events in experimental mouse models (28). In a similar model, the dedicator of cytokinesis protein 4 (DOCK4) was also identified as another potential biomarker of BM. This preclinical result was also validated by tissue microarray from the large AZURE adjuvant study (20). In the control group, higher DOCK4 expression was significantly prognostic for first bone distant recurrence, whereas in the zoledronic group, this association was lost, suggesting that treatment with zoledronate may counteract the higher risk for bone relapse from high DOCK4-expressing tumors (20). Importantly, DOCK4 expression was not associated with risk of non-skeletal events (20).

Additional candidate biomarkers have been recently identified as predictors of metastatic spread to the bone: among these, nuclear p21-activated kinase 4 (nPAK4) expression was associated with BM development specifically in estrogen receptor alpha (ERα) positive BCa patients via targeting of the leukemia inhibitory factor receptor (LIFR), a BM suppressor (21). Other osteoclastogenesis mediators, including peroxiredoxin-4 (PRDX4) and L-plastin (LPC1), have been identified as responsible for tumor bone colonization in a number of osteotropic cancers such as breast, prostate, and renal cancers (22). Furthermore, an association between increased levels of circulating prolactin (PRL) and BCa metastases has been reported (23, 24), and recent studies showed that high expression of the PRL receptor (PRLR) on a primary tumor correlated with a shorter time to BM (25).

Recently, Li et al. (26) identified a panel of 51 genes differentially expressed between non-metastatic and bone metastatic BCa patients, starting from a merged data set containing clinical and transcriptomic data of 855 BCa patients. The panel validated by survival analyses showed a high performance in predicting BM. Similarly, Zhao et al. (27) developed a gene expression signature-based nomogram model to predict BM in BCa patients. In particular, using three microarray data sets of 572 patients, including 191 with BM and 381 metastases-free, they identified five BM-related genes: keratin 23 (KRT23), receptor accessory protein 1 (REEP1), spi-B transcription factor (SPIB), aldehyde dehydrogenase 3 family member B2 (ALDH3B2), and glycine decarboxylase (GLDC). These genes were then used to set up a model able to identify bone recurrence with high predictive power (with a C-index of 0.677 for the training set and 0.689 and 0.695 for the testing sets, respectively).

Although this and other models could represent useful prediction tools for the clinicians, most of the biomarkers derived from protein and gene expression profiles do not currently have

Abbreviations: ALDH3B2, Aldehyde dehydrogenase 3 family member B2; BCa, breast cancer; BM, bone metastases; BTM, bone turnover markers; CTX, C-telopeptide of type-1 collagen; CXCR4, C-X-C chemokine receptor 4; CRPC, castration-resistant prostate cancer; CS, CellSearch; CTCs, circulating tumor cells; ctDNA, circulating tumor DNA; DOCK4, dedicator of cytokinesis protein 4; DTCs, disseminated tumor cells; EpCAM, epithelial cell adhesion molecule; EMT, epithelial–mesenchymal transition; ERα, estrogen receptor alpha; GIPC1, GIPC PDZ domain containing family, member 1; GLDC, glycine decarboxylase; HCC, hepatocellular carcinoma; ITGBL1, integrin beta-like 1; IL-1β, interleukin-1β; KRT23, keratin 23; LPC1, L-plastin; LIFR, leukemia inhibitory factor receptor; LC, lung cancer; CAPG, macrophage-capping protein; miRNAs, microRNAs; P1NP, N-terminal propeptide of type-1 collagen; NTX, N-terminal telopeptide; NET, neuroendocrine tumors; NSCLC, non-small cell lung cancer; nPAK4, nuclear p21-activated kinase 4; RANK, nuclear-factor–κB; OPG, osteoprotegerin; OS, overall survival; PRDX4, peroxiredoxin-4; PCR, polymerase chain reaction; PRLR, PRL receptor; PRL, prolactin; PCa, prostate cancer; 1-CTP, pyridinoline cross-linked carboxy-terminal telopeptide of type-1 collagen; REEP1, receptor accessory protein 1; RANKL, receptor activator of nuclear factor κB ligand; RUNX2, runt-related transcription factor 2; GESBN, signature-based nomogram; SPIB, Spi-B transcription factor; TRAcP-5b, tartrate-resistant acid phosphatase 5b; TCGA, The Cancer Genome Atlas; TMA, tissue microarray; Tgif2, transforming growth factor-β-induced factor 2; TFF1, Trefoil factor 1.

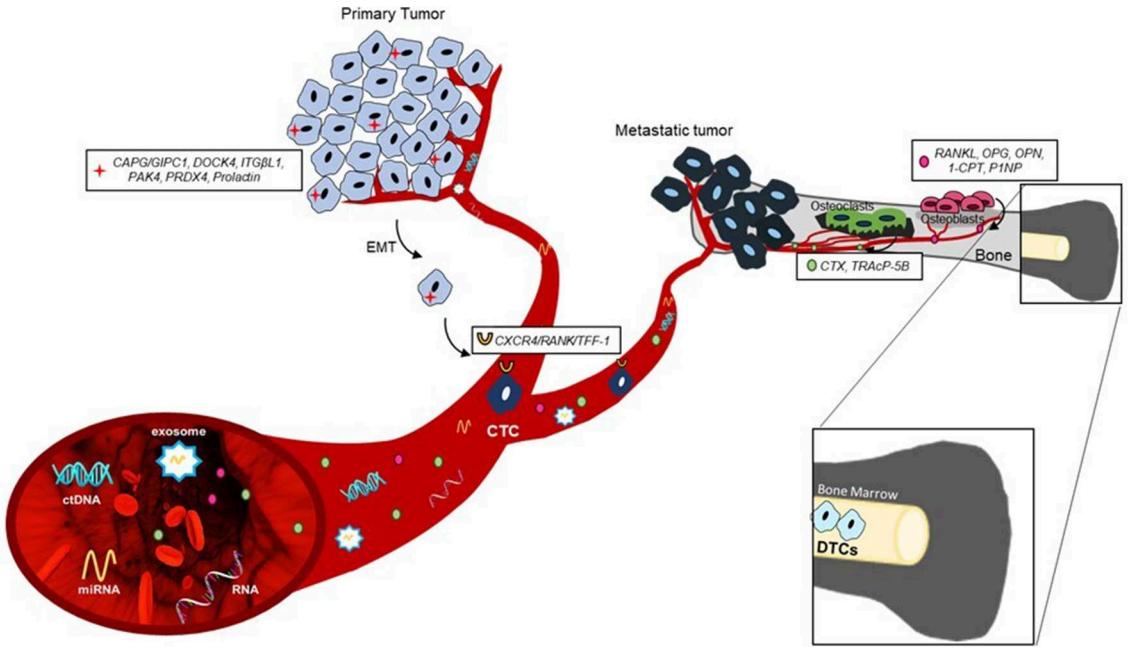

FIGURE 1 | Principal predictive biomarkers of bone metastasis development.

TABLE 1 | Expression gene profiles predicting bone metastases.

Biomarker	Tumor type	Predictive role in bone metastasis	References
CAPG, GIPC1	Breast cancer	High expression levels	(17)
ITGBL1	Breast cancer	Coexpressed with other genes related to osteomimicry	(18)
IL-1 β	Breast cancer	High expression levels	(19)
DOCK-4	Breast cancer	High levels of expression	(20)
nPAK4	Breast cancer	Elevated gene expression	(21)
PRDX4, LPC1	Breast, prostate, and renal cancers	High levels of expression	(22)
PRL, PRLR	Breast cancer	Elevated gene expression	(23–25)
GESBN model	Breast cancer	A panel of 51 genes predict bone recurrence	(26, 27)

standardized analytical tools to be measured and therefore have not been sufficiently validated to be widely adopted.

ROLE OF CIRCULATING TUMOR CELLS AND DISSEMINATED TUMOR CELLS IN PREDICTING BONE RECURRENCE

Circulating tumor cells (CTCs) are defined as cancer cells originating from primary and/or metastatic sites and circulating in the bloodstream. CTCs have shown prognostic implications in a variety of cancer types, including BCa, prostate cancer (PCa), non-small cell lung cancer (NSCLC), colorectal cancer, and others (29). CTCs provide clinical relevant information about tumor burden, biological aggressiveness of the disease, the presence of undetectable micrometastases, and the tendency to metastatic spread.

Several evidences suggest that CTCs count can be used as an early predictor of bone metastatic potential in PCa (30), BCa (31), and NSCLC (32) (**Table 2**). In particular, in castration-resistant prostate cancer (CRPC) patients, CTC detection was closely associated with the clinical evidence of BM and with survival (30). Similarly, a higher CTC number were detected in patients with BCa with BM relative to those with no bone lesions, and in patients with multiple bone metastases relative to those with one or two bone lesions (31). Higher baseline CTC count was also predictive of BM development in lung cancer (LC) patients (32).

In addition, the molecular characterization of CTCs may carry relevant biological information regarding the heterogeneity of the metastatic disease. Wang et al. identified a gene profile in circulating BCa cells significantly associated with BM presence. This signature showed that trefoil factor 1 (TFF1) was the most correlated gene with BM onset (39). Another study reported a strong association in the expression of several genes related to

TABLE 2 | Predictive role of CTCs and DTCs in bone metastasis onset.

Biomarker	Tumor type	Predictive role in bone metastasis	References
CTCs	Prostate, breast, and lung cancers	High CTC count	(30–32)
CTCs	Breast cancer	TFF-1 expression on CTCs	(33)
CTCs	Breast cancer	RANK expression on CTCs	(34)
CTCs	Neuroendocrine tumors	CXCR4 expression on CTCs	(35)
DTCs	Breast cancer	High DTC count	(36)
DTCs	Breast cancer	Postoperative presence of DTCs	(7)
DTCs	Prostate cancer	DTC presence at baseline	(37, 38)

TABLE 3 | Predictive role of ctDNA and miRNA in bone metastasis onset.

Biomarker	Tumor type	Predictive role in bone metastasis	References
ctDNA	Lung cancer	Presence of ctDNA at baseline	(45)
ctDNA	Lung cancer	Higher ctDNA levels	(46)
ctDNA	Gastrointestinal, brain, lung, breast, and others	Presence of MET alterations	(47)
miRNA	Breast cancer	miR-19a, miR-93, miR-106a score	(48)
miRNA	Hepatocellular carcinoma	miR-34a reduced serum levels	(49)
miRNA	Breast cancer	miR-30 family low expression in primary tissue	(50)
miRNA	Prostate cancer	miR-466 low expression	(51)
miRNA	Breast cancer	miR-135 and miR-203 absence in metastatic tissues	(52)

disease progression and therapy resistance between CTCs and bone metastatic tissue of PCa patients (33).

These evidences support a potential role of CTC phenotyping as a tool to predict BM onset. In this regard, we recently identified a receptor activator of nuclear-factor–κB (RANK)-positive CTC in bone metastatic BCa patients, suggesting that RANK expression may represent a phenotypic and biologic property of cancer cells with elevated osteotropism (34). This is further supported by the evidence of a strong correlation between high RANK expression in BCa as well as other primary tumor types and BM relapse (40, 41).

CTC presence is associated with BM also in patients affected by neuroendocrine tumors (NETs) (35). Interestingly, in these patients, a high percentage of CTCs expressed C-X-C chemokine receptor 4 (CXCR4), a well-known molecule involved in osteotropism (35).

Besides CTCs, several evidences have shown an association between presence of DTCs and BM occurrence in stage IV BCa. Moreover, a higher frequency of DTCs was observed in patients with lobular carcinoma, the histotype that most frequently spread to bone, compared with ductal carcinoma (36). A pooled prospective analysis of more than 4,000 BCa patients demonstrated that DTC identification in bone marrow predicted postoperative disease recurrence, including BM (7). Similarly, DTC count in bone marrow aspirates of PCa patients, collected before the initiation of primary therapy, was an independent prognostic factor of patients' survival and bone relapse (37, 42). It is well-established that the persistence of DTCs during follow-up is associated with a shorter relapse-free survival and poorer prognosis (38, 43). Interestingly, the presence of DTCs in the bone marrow is a predictor of bone-specific recurrence and could be used to identify patients with high risk to develop skeletal disease (**Table 2**).

CIRCULATING TUMOR DNA AND MIRNAS AS NON-INVASIVE BIOMARKER FOR BONE METASTASES PREDICTION

In the last few years, several studies demonstrated the potential clinical utility of circulating tumor DNA (ctDNA) both in the early diagnosis of tumors and in the monitoring of therapeutic efficacy. ctDNA contains tumor-specific genetic and epigenetic alterations, which makes it a useful non-invasive prognostic and predictive biomarker in different solid tumors (44). A number of studies support the idea that ctDNA levels might be predictors of BM development (**Table 3**). In particular, the presence of ctDNA at baseline was associated with BM in newly diagnosed patients with advanced NSCLC (45). Similar results were obtained in late-stage NSCLC patients in which higher levels of ctDNA were associated to BM presence (46). A recent study demonstrated that MET alterations detected in ctDNA correlated with BM affected by different solid tumors (47). Since MET is greatly expressed in the bone microenvironment (53), it is therefore conceivable that the high rates of ctDNA bearing MET alterations derive from secondary bone lesions. Therefore, ctDNA profiling could represent an excellent tool to detect these specific alterations and anticipate bone metastatic recurrence prior to clinical detection.

MicroRNAs (miRNAs) are endogenous non-coding small RNAs that play a key role in various biological processes including bone remodeling (54, 55). Thanks to their high stability in blood, miRNAs have become promising biomarker candidates

TABLE 4 | Bone turnover markers predicting bone relapse.

Biomarker	Tumor type	Predictive role in bone metastasis	References
P1NP, CTX and 1-CTP	Breast cancer	High serum levels	(63)
CTX	Breast cancer	High serum levels	(64)
Vitamin D	Breast cancer	Vit D deficiency	(65)
P1NP	Prostate cancer	High serum levels	(66)
TRAcP-5b	Prostate and Breast cancers	High serum levels	(67, 68)
OPG/RANKL	Prostate cancer	Alteration of OPG/RANKL balance	(69, 70)
Osteopontin	Renal carcinoma	High serum levels	(71, 72)
NTX, P1NP, CTX, 1-CTP, TRAcP-5b	Lung cancer	High serum levels	(73–78)

for cancer detection and monitoring, predicting outcomes and chemoresistance. Several evidences have shown a possible role of miRNAs as novel specific biomarkers of bone recurrence (**Table 3**). Recently, a three-miRNA signature score, which includes miR-19a, miR-93, and miR-106a, has been identified as a predictor of BM occurrence in BCa using The Cancer Genome Atlas (TCGA) datasets (48). It would be important to validate their expression levels in early BCa patients to assess their ability to predict the BM onset.

A miRNA microarray analysis in hepatocellular carcinoma (HCC) patients, with and without skeletal disease, showed that serum miR-34a expression levels were independent predictors of BM development (49). Previous evidences reported a critical role of miR-34a as a suppressor of osteoclastogenesis and bone resorption through the targeting of transforming growth factor-β-induced factor 2 (Tgif2) (56).

Recently, Croset et al. demonstrated a direct involvement of miR-30 family members in promoting BCa BM *in vitro* and *in vivo*. In addition, they found that low expression of miR-30 in primary tumors was correlated with poor relapse-free survival (50). Serum analyses of miR-30 members in a prospective trial of non-metastatic BCa patients could give a further confirmation of their predictive value in the early detection of BM.

The microRNA miR-466 has been significantly associated with BM development in PCa (51). In xenograft models, miR-466 overexpression interrupts runt-related transcription factor 2 (RUNX2) integrated network of genes preventing BM. In addition, miR-466 expression in primary tissue also predicted biochemical relapse, suggesting its clinical significance in bone metastatic process (51). The other two RUNX2-targeting microRNAs, miR-135 and miR-203, were associated to BCa growth in bone (52). In particular, these miRNAs were absent in BM expressing high levels of RUNX2, suggesting their fundamental role in regulating tumor osteotropism mediated by RUNX2 (52). Since RUNX2 represents a key player of bone metastatic process, the detection of RUNX2-targeting microRNAs in the blood could be extremely useful to monitor and control skeletal disease progression.

More recently, exosomal miRNAs have emerged as important regulators of BM in preclinical studies (57). It is well-established that tumor-derived exosomes can affect bone remodeling promoting the vicious cycle of BM (58). So far, only a few studies reported a correlation between specific exosomal miRNAs and bone metastases. Valencia et al. demonstrated that exosomes carrying miR-192 reduced metastatic bone colonization (59); on the contrary, Hashimoto et al. found high levels of specific miRNAs in exosomes of PCa cells with elevated propensity to metastasize into the skeleton (60). Considering the accumulating evidences regarding the role of exosomal miRNAs in cancer, this area of investigation should be further developed.

CHANGES IN BIOCHEMICAL MARKERS OF BONE TURNOVER PREDICT BONE METASTASES

Biochemical markers of bone metabolism reflect the bone turnover, and variations in their levels have been correlated with BM onset and their complications (61, 62) (**Table 4**). The determination of bone markers in the serum and/or urine could provide a non-invasive procedure that is helpful in predicting and monitoring the progression of disease into the skeleton. Alteration of these markers reflects specific changes in bone microenvironment, which becomes a fertile niche for tumor cell homing.

Patients with high serum levels of N-terminal propeptide of type-1 collagen (P1NP), C-telopeptide of type-1 collagen (CTX), and pyridinoline cross-linked carboxy-terminal telopeptide of type-1 collagen (1-CTP) after diagnosis were shown to be at high risk for bone recurrence, but not for other metastatic sites. In addition, none of these markers was predictive of treatment benefit from zoledronic acid (63).

Moreover, in the NCIC CTG MA.14 study, a high CTX serum level correlated with bone-only relapse probably due to an increased bone metabolism that may facilitate the development of skeletal metastasis (64). Conversely, any correlation between high CTX-I and P1NP levels and bone relapse was found (65), but, surprisingly, normal levels of serum vitamin D were associated with a lower risk of BM occurrence.

Several studies have reported strong correlations between elevated levels of bone turnover markers (BTMs) and the presence and the extent of skeletal disease in PCa (79, 80). Interestingly, increased P1NP levels identified PCa patients with BM vs. lymph node metastases before the first positive bone scintigraphy (66). Other studies identified significant associations between elevated plasma levels of tartrate-resistant

acid phosphatase 5b (TRAcP-5b) (67, 68), osteoprotegerin (OPG) (69, 70), and osteopontin, and presence of BM in PCa and renal cancer patients (71, 72). Similarly, serum levels of BTM [such as N-terminal telopeptide (NTX), CTX, TRAcP-5b, P1NP] are strongly associated with the development and progression of BM in patients with LC (73–78).

Overall, these evidences highlighted the potential role of BTM as predictors of BM occurrence in different solid tumors.

DISCUSSION

The identification of patients at risk for BM could offer the opportunity to treat them at an earlier stage, improving their clinical outcomes.

In the last decades, genomic and proteomic analyses have led to the identification of molecular signatures on tumor tissue that predict bone relapse with sufficient accuracy. Indeed, several tissue biomarkers have been identified as predictive for BM development, including the composite CAPG/GIPC1 proteins and DOCK4, with the latter clinically validated. In addition, the emerging use of computational models to generate predictive signatures has significantly grown in the last years thanks to the availability of high-throughput datasets and novel data analysis tools.

More recently, liquid biopsy has emerged as a rapid, noninvasive source of biomarkers including CTCs, DTCs, ctDNA, and circulating miRNA. Liquid biopsy has the strong advantage to overcome tumor heterogeneity and capture the changing and evolving landscape of cancer in real time during the course of the disease. The molecular characterization of CTCs showed that the expression of osteotropic markers such as RANK and CXCR4 could be responsible for tumor cell homing to the bone. Thus, CTC phenotyping could dynamically track changes in tumor cell profile and predict their migration into the skeleton. Several procedures have been developed in the last decades for CTC isolation and detection, but so far the Food and Drug Administration has approved CellSearch (CS) as the unique platform for CTC enumeration. Nevertheless, CTC identification by CS based on biological characteristics (e.g., the expression of the epithelial markers such as epithelial cell adhesion molecule, EpCAM, and cytokeratins) does not reach 100% of sensitivity and specificity. For example, patients with epithelial cancers might present CTCs expressing mesenchymal rather than epithelial markers, as a result of epithelial-to-mesenchymal transition, a phenomenon associated to disease progression (81–83). These technical limitations have slowed the diagnostic and prognostic use of CTC blood test into clinical practice. DTCs have been demonstrated to be strong predictors of BM onset in both early BCa and PCa. Similar to CTCs, also DTC detection and analysis present some technical limitations including a low number of cells and the difficulty to characterize them with standard technologies such as flow cytometry, immunofluorescence, or polymerase chain reaction (PCR). Moreover, BM aspiration procedure is an invasive method that cannot be repeated unlimitedly.

Since the release of ctDNA into the bloodstream is frequently in cancer patients, screening of ctDNA may provide clinically relevant information about mutational profiles associated with BM development. There are still many challenges that need to be overcome before its introduction in clinical practice. Due to the extremely low levels in the blood, ctDNA sensitivity and specificity remain the principal issues. Current digital PCR methods fail to detect smaller fragments derived from tumors increasing false negative, but advances in genomic approaches could allow us to identify all ctDNA in the blood. Due to their high stability in the blood, circulating miRNAs are probably the most promising biomarkers of bone recurrence. Indeed, several miRNAs have been identified as key regulators of the principal genes involved in bone remodeling and cancer bone tropism. The development of different technical platforms over other RNA-seq technologies guarantees an intrinsic technical reproducibility needed for their rapid translation in clinical practice.

Finally, BTM could represent easily measured factors that are able to predict BM in patients with early stage of cancer. Indeed, P1NP, CTX, and 1-CTP were found to be predictive of bone-specific recurrence, suggesting that an increased bone turnover creates a fertile environment that promotes cancer cell adhesion and growth. Nevertheless, BTM levels can be influenced not only by patients' features, such as age, sex, and food intake, but also by systemic treatments that affect bone remodeling.

AUTHOR CONTRIBUTIONS

DS and MI contributed conception and design of the study. SS and MI wrote the first draft of the manuscript. GR, SS, and FP performed figure and tables of the manuscript. DS, GT, BV, and FP revised the manuscript. All authors contributed to read and approved the submitted version.

REFERENCES

1. Coleman RE. Skeletal complications of malignancy. *Cancer*. (1997) 80(8 Suppl.):1588–94. doi: 10.1002/(sici)1097-0142(19971015)80:8+ <1588::aid-cncr9>3.3.co;2-z
2. Meads MB, Hazlehurst LA, Dalton WS. The bone marrow microenvironment as a tumor sanctuary and contributor to drug resistance. *Clin Cancer Res*. (2008) 14:2519–26. doi: 10.1158/1078-0432.CCR-07-2223
3. Shiozawa Y, Havens AM, Pienta KJ, Taichman RS. The bone marrow niche: habitat to hematopoietic and mesenchymal stem cells, and unwitting host to molecular parasites. *Leukemia*. (2008) 22:941–50. doi: 10.1038/leu.2008.48
4. Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer*. (2002) 2:563–72. doi: 10.1038/nrc865
5. Bellahcene A, Bachelier R, Detry C, Lidereau R, Clezardin P, Castronovo V. Transcriptome analysis reveals an osteoblast-like phenotype for human osteotropic breast cancer cells. *Breast Cancer Res Treat*. (2007) 101:135–48. doi: 10.1007/s10549-006-9279-8

6. Bidard FC, Vincent-Salomon A, Gomme S, Nos C, de Rycke Y, Thiery JP, et al. Disseminated tumor cells of breast cancer patients: a strong prognostic factor for distant and local relapse. *Clin Cancer Res*. (2008) 14:3306–11. doi: 10.1158/1078-0432.CCR-07-4749
7. Braun S, Vogl FD, Naume B, Janni W, Osborne MP, Coombes RC, et al. A pooled analysis of bone marrow micrometastasis in breast cancer. *N Engl J Med*. (2005) 353:793–802. doi: 10.1056/NEJMoa050434
8. Cristofanilli M, Hayes DF, Budd GT, Ellis MJ, Stopeck A, Reuben JM, et al. Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer. *J Clin Oncol*. (2005) 23:1420–30. doi: 10.1200/JCO.2005.08.140
9. Jacobs C, Amir E, Paterson A, Zhu X, Clemons M. Are adjuvant bisphosphonates now standard of care of women with early stage breast cancer? A debate from the Canadian bone and the oncologist new updates meeting. *J Bone Oncol*. (2005) 4:54–8. doi: 10.1016/j.jbo.2015.06.001
10. Gnant M, Mlineritsch B, Stoeger H, Luschin-Ebengreuth G, Knauer M, Moik M et al. Zoledronic acid combined with adjuvant endocrine therapy of tamoxifen versus anastrozol plus ovarian function suppression in premenopausal early breast cancer: final analysis of the Austrian breast and colorectal cancer study group trial 12. *Ann Oncol*. (2015) 26:313–20. doi: 10.1093/annonc/mdu544
11. Coleman R, de Boer R, Eidtmann H, Llombart A, Davidson N, Neven P, et al. Zoledronic acid (zoledronate) for postmenopausal women with early breast cancer receiving adjuvant letrozole (ZO-FAST study): final 60-month results. *Ann Oncol*. (2013) 24:398–405. doi: 10.1093/annonc/mds277
12. von Minckwitz G, Möbus V, Schneeweiss A, Huober J, Thomssen C, Untch M, et al. German adjuvant intergroup node-positive study: a phase III trial to compare oral ibandronate versus observation in patients with high-risk early breast cancer. *J Clin Oncol*. (2013) 31:3531–9. doi: 10.1200/JCO.2012.47.2167
13. Paterson AH, Anderson SJ, Lembersky BC, Fehrenbacher L, Falkson CI, King KM, et al. Oral clodronate for adjuvant treatment of operable breast cancer (National surgical adjuvant breast and bowel project protocol B-34): a multicentre, placebo-controlled, randomised trial. *Lancet Oncol*. (2012) 13:734–42. doi: 10.1016/S1470-2045(12)70226-7
14. Early Breast Cancer Trialists' Collaborative Group. Adjuvant bisphosphonate treatment in early breast cancer: meta-analyses of individual patient data from randomised trials. *Lancet*. (2015) 386:1353–61. doi: 10.1016/S0140-6736(15)60908-4
15. Gnant M, Pfeiler G, Steger GG, Egle D, Greil R, Fitzal F, et al. Adjuvant denosumab in postmenopausal patients with hormone receptor-positive breast cancer (ABCSG-18): disease-free survival results from a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncol*. (2019) 20:339–51. doi: 10.1016/S1470-2045(18)30862-3
16. Coleman R, Finkelstein DM, Barrios C, Martin M, Iwata H, Hegg R, et al. Adjuvant denosumab in early breast cancer (D-CARE): an international, multicentre, randomised, controlled, phase 3 trial. *Lancet Oncol*. (2020) 21:60–72. doi: 10.1016/S1470-2045(19)30687-4
17. Westbrook JA, Cairns DA, Peng J, Speirs V, Hanby AM, Holen I, et al. CAPG and GIPC1: breast cancer biomarkers for bone metastasis development and treatment. *J Natl Cancer Inst*. (2016) 108:djv360. doi: 10.1093/jnci/djv360
18. Li XQ, Du X, Li DM, Kong PZ, Sun Y, Liu PF, et al. ITGBL1 is a Runx2 transcriptional target and promotes breast cancer bone metastasis by activating the TGFβ signaling pathway. *Cancer Res*. (2015) 75:3302–13. doi: 10.1158/0008-5472.CAN-15-0240
19. Nutter F, Holen I, Brown HK, Cross SS, Evans CA, Walker M, et al. Different molecular profiles are associated with breast cancer cell homing compared with colonisation of bone: evidence using a novel bone-seeking cell line. *Endocr Relat Cancer*. (2014) 21:327–41. doi: 10.1530/ERC-13-0158
20. Westbrook JA, Wood SL, Cairns DA, McMahon K, Gahlaut R, Thygesen H, et al. Identification and validation of DOCK4 as a potential biomarker for risk of bone metastasis development in patients with early breast cancer. *J Pathol*. (2019) 247:381–91. doi: 10.1002/path.5197
21. Li Y, Zhang H, Zhao Y, Wang C, Cheng Z, Tang L, et al. A mandatory role of nuclear PAK4-LIFR axis in breast-to-bone metastasis of ERα-positive breast cancer cells. *Oncogene*. (2019) 38:808–21. doi: 10.1038/s41388-018-0456-0
22. Tiedemann K, Sadvakassova G, Mikolajewicz N, Juhas M, Sabirova Z, Tabariès S, et al. Exosomal release of L-plastin by breast cancer cells facilitates metastatic bone osteolysis. *Transl Oncol*. (2019) 12:462–74. doi: 10.1016/j.tranon.2018.11.014
23. Tikk K, Sookthai D, Johnson T, Rinaldi S, Romieu I, Tjønneland A, et al. Circulating prolactin and breast cancer risk among pre- and postmenopausal women in the EPIC cohort. *Ann Oncol*. (2014) 25:1422–8. doi: 10.1093/annonc/mdu150
24. Miller SL, Antico G, Raghunath PN, Tomaszewski JE, Clevenger CV. Nek3 kinase regulates prolactin-mediated cytoskeletal reorganization and motility of breast cancer cells. *Oncogene*. (2007) 26:4668–78. doi: 10.1038/sj.onc.1210264
25. Sutherland A, Forsyth A, Cong Y, Grant L, Juan TH, Lee JK, et al. The role of prolactin in bone metastasis and breast cancer cell-mediated osteoclast differentiation. *J Natl Cancer Inst*. (2015) 108:djv338. doi: 10.1093/jnci/djv338
26. Li JN, Zhong R, Zhou XH. Prediction of bone metastasis in breast cancer based on minimal driver gene set in gene dependency network. *Genes*. (2019) 10:E466. doi: 10.3390/genes10060466
27. Zhao C, Lou Y, Wang Y, Wang D, Tang L, Gao X, et al. A gene expression signature-based nomogram model in prediction of breast cancer bone metastases. *Cancer Med*. (2019) 8:200–8. doi: 10.1002/cam4.1932
28. Holen I, Lefley DV, Francis SE, Rennicks S, Bradbury S, Coleman RE, et al. IL-1 drives breast cancer growth and bone metastasis *in vivo*. *Oncotarget*. (2016) 7:75571–584. doi: 10.18632/oncotarget.12289
29. Sundling KE, Lowe AC. Circulating tumor cells. Overview and opportunities in cytology. *Adv Anat Pathol*. (2019) 26:56–63. doi: 10.1097/PAP.0000000000000217
30. Helo P, Cronin AM, Danila DC, Wenske S, Gonzalez-Espinoza R, Anand A, et al. Circulating prostate tumor cells detected by reverse transcription-PCR in men with localized or castration-refractory prostate cancer: concordance with CellSearch assay and association with bone metastases and with survival. *Clin Chem*. (2009) 55:765–73. doi: 10.1373/clinchem.2008.117952
31. De Giorgi U, Valero V, Rohren E, Mego M, Doyle GV, Miller MC, et al. Circulating tumor cells and bone metastases as detected by FDG-PET/CT in patients with metastatic breast cancer. *Ann Oncol*. (2010) 21:33–9. doi: 10.1093/annonc/mdp262
32. Cheng M, Liu L, Yang HS, Liu GF. Circulating tumor cells are associated with bone metastasis of lung cancer. *Asian Pac J Cancer Prev*. (2014) 15:6369–74. doi: 10.7314/APJCP.2014.15.15.6369
33. Josefsson A, Larsson K, Månsson M, Björkman J, Rohlova E, Åhs D, et al. Circulating tumor cells mirror bone metastatic phenotype in prostate cancer. *Oncotarget*. (2018) 9:29403–13. doi: 10.18632/oncotarget.25634
34. Pantano F, Rossi E, Iuliani M, Facchinetti A, Simonetti S, Ribelli G, et al. Dynamic changes of receptor activator of nuclear factor-κB expression in circulating tumor cells during denosumab predict treatment effectiveness in metastatic breast cancer, *Sci Rep*. (2020) 10:1288. doi: 10.1038/s41598-020-58339-2
35. Rizzo FM, Vesely C, Childs A, Marafioti T, Khan MS, Mandair D, et al. Circulating tumour cells and their association with bone metastases in patients with neuroendocrine tumours. *Br J Cancer*. (2019) 120:294–300. doi: 10.1038/s41416-018-0367-4
36. Bidard FC, Vincent-Salomon A, Sigal-Zafrani B, Diéras V, Mathiot C, Mignot L, et al. Prognosis of women with stage IV breast cancer depends on detection of circulating tumor cells rather than disseminated tumor cells. *Ann Oncol*. (2008) 19:496–500. doi: 10.1093/annonc/mdm507
37. Morgan TM, Lange PH, Porter MP, Lin DW, Ellis WJ, Gallaher IS, et al. Disseminated tumor cells in prostate cancer patients after radical prostatectomy and without evidence of disease predicts biochemical recurrence. *Clin Cancer Res*. (2009) 15:677–83. doi: 10.1158/1078-0432.CCR-08-1754
38. Janni W, Vogl FD, Wiedswang G, Synnestvedt M, Fehm T, Jückstock J, et al. Persistence of disseminated tumor cells in the bone marrow of breast cancer patients predicts increased risk for relapse–a European pooled analysis. *Clin Cancer Res*. (2011) 17:2967–76. doi: 10.1158/1078-0432.CCR-10-2515
39. Wang H, Molina J, Jiang J, Ferber M, Pruthi S, Jatkoe T, et al. Gene expression markers in circulating tumor cells may predict bone metastasis and response to hormonal treatment in breast cancer. *Mol Clin Oncol*. (2013) 1:1031–8. doi: 10.3892/mco.2013.163
40. Santini D, Schiavon G, Vincenzi B, Gaeta L, Pantano F, Russo A, et al. Receptor activator of NF-kB (RANK) expression in primary tumors associates

with bone metastasis occurrence in breast cancer patients. *PLoS ONE.* (2011) 6:e19234. doi: 10.1371/journal.pone.0019234
41. Santini D, Perrone G, Roato I, Godio L, Pantano F, Grasso D, et al. Expression pattern of receptor activator of NFκB (RANK) in a series of primary solid tumors and related bone metastases. *J Cell Physiol.* (2011) 226:780–4. doi: 10.1002/jcp.22402
42. Lilleby W, Stensvold A, Mills IG, Nesland JM. Disseminated tumor cells and their prognostic significance in nonmetastatic prostate cancer patients. *Int J Cancer.* (2013) 133:149–55 doi. 10.1002/ijc.28002.doi: 10.1002/ijc.28002
43. Mansi JL, Gogas H, Bliss JM, Gazet JC, Berger U, Coombes RC. Outcome of primary-breast-cancer patients with micrometastases: a long-term follow-up study. *Lancet.* (1999) 354:197–202. doi: 10.1016/S0140-6736(98)10175-7
44. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med.* (2014) 6:224ra24. doi: 10.1158/1538-7445.AM2014-5606
45. Pecuchet N, Zonta E, Didelot A, Combe P, Thibault C, Gibault L, et al. Base-position error rate analysis of next-generation sequencing applied to circulating tumor DNA in non-small cell lung cancer: a prospective study. *PLoS Med.* (2016) 13:e1002199. doi: 10.1371/journal.pmed.1002199
46. Jia J, Huang B, Zhuang Z, Chen S. Circulating tumor DNA as prognostic markers for late stage NSCLC with bone metastasis. *Int J Biol Markers.* (2018) 33:222–30. doi: 10.1177/1724600817753576
47. Ikeda S, Schwaederle M, Mohindra M, Fontes Jardim DL, Kurzrock R. MET alterations detected in blood-derived circulating tumor DNA correlate with bone metastases and poor prognosis. *J Hematol Oncol.* (2018) 11:76. doi: 10.1186/s13045-018-0610-8
48. Kawaguchi T, Yan L, Qi Q, Peng X, Edge SB, Young J, et al. Novel MicroRNA-based risk score identified by integrated analyses to predict metastasis and poor prognosis in breast cancer. *Ann Surg Oncol.* (2018) 25:4037–46. doi: 10.1245/s10434-018-6859-x
49. Xiang ZL, Zhao XM, Zhang L, Yang P, Fan J, Tang ZY, et al. Correction: MicroRNA-34a expression levels in serum and intratumoral tissue can predict bone metastasis in patients with hepatocellular carcinoma. *Oncotarget.* (2019) 10:6398–9. doi: 10.18632/oncotarget.27296
50. Croset M, Pantano F, Kan CWS, Bonnelye E, Descotes F, Alix-Panabières C, et al. miRNA-30 family members inhibit breast cancer invasion, osteomimicry, and bone destruction by directly targeting multiple bone metastasis-associated genes. *Cancer Res.* (2018) 78:5259–73. doi: 10.1158/0008-5472.CAN-17-3058
51. Colden M, Dar AA, Saini S, Dahiya PV, Shahryari V, Yamamura S, et al. MicroRNA-466 inhibits tumor growth and bone metastasis in prostate cancer by direct regulation of osteogenic transcription factor RUNX2. *Cell Death Dis.* (2017) 8:e2572. doi: 10.1038/cddis.2017.15
52. Taipaleenmäki H, Browne G, Akech J, Zustin J, van Wijnen AJ, Stein JL, et al. Targeting of Runx2 by miR-135 and miR-203 impairs progression of breast cancer and metastatic bone disease. *Cancer Res.* (2015) 75:1433–44. doi: 10.1158/0008-5472.CAN-14-1026
53. Fioramonti M, Santini D, Iuliani M, Ribelli G, Manca P, Papapietro N, et al. Cabozantinib targets bone microenvironment modulating human osteoclast and osteoblast functions. *Oncotarget.* (2017) 8:20113–21. doi: 10.18632/oncotarget.15390
54. Lian JB, Stein GS, van Wijnen AJ, Stein JL, Hassan MQ, Gaur T, et al. MicroRNA control of bone formation and homeostasis. *Nat Rev Endocrinol.* (2012) 8:212–27. doi: 10.1038/nrendo.2011.234
55. Drusco A, Croce CM. MicroRNAs and cancer: a long story for short RNAs. *Adv Cancer Res.* (2017) 135:1–24. doi: 10.1016/bs.acr.2017.06.005
56. Krzeszinski JY, Wei W, Huynh H, Jin Z, Wang X, Chang TC, et al. miR-34a blocks osteoporosis and bone metastasis by inhibiting osteoclastogenesis and Tgif2. *Nature.* (2014) 512:431–5. doi: 10.1038/nature13375
57. Probert C, Dottorini T, Speakman A, Hunt S, Nafee T, Fazeli A, et al. Communication of prostate cancer cells with bone cells via extracellular vesicle RNA; a potential mechanism of metastasis. *Oncogene.* (2019) 38:1751–63. doi: 10.1038/s41388-018-0540-5
58. Zhao H, Achreja A, Iessi E, Logozzi M, Mizzoni D, Di Raimo R, et al. The key role of extracellular vesicles in the metastatic process. *Biochim Biophys Acta Rev Cancer.* (2018) 1869:64–77. doi: 10.1016/j.bbcan.2017.11.005
59. Valencia K, Luis-Ravelo D, Bovy N, Anton I, Martinez-Canarias S, Zandueta C, et al. miRNA cargo within exosome-like vesicle transfer influences metastatic bone colonization. *Mol Oncol.* 8:689–703. doi: 10.1016/j.molonc.2014.01.012
60. Hashimoto K, Ochi H, Sunamura S, Kosaka N, Mabuchi Y, Fukuda T, et al. Cancer-secreted hsa-miR-940 induces an osteoblastic phenotype in the bone metastatic microenvironment via targeting ARHGAP1 and FAM134A. *Proc Natl Acad Sci USA.* (2018) 115:2204–9. doi: 10.1073/pnas.1717363115
61. Jung K, Lein M. Bone turnover markers in serum and urine as diagnostic, prognostic and monitoring biomarkers of bone metastasis. *Biochim Biophys Acta.* (2014) 1846:425–38. doi: 10.1016/j.bbcan.2014.09.001
62. Seibel MJ. Clinical use of markers of bone turnover in metastatic bone disease. *Nat Clin Pract Oncol.* (2005) 2:504–17. doi: 10.1038/ncponc0320
63. Brown J, Rathbone E, Hinsley S, Gregory W, Gossiel F, Marshall H, et al. Associations between serum bone biomarkers in early breast cancer and development of bone metastasis: results from the AZURE (BIG01/04) trial. *J Natl Cancer Inst.* (2018) 110:871–9. doi: 10.1093/jnci/djx280
64. Lipton A, Chapman JA, Demers L, Shepherd LE, Han L, Wilson CF, et al. Elevated bone turnover predicts for bone metastasis in postmenopausal breast cancer: results of NCIC CTG MA.14. *J Clin Oncol.* (2011) 29:3605–10. doi: 10.1200/JCO.2010.31.5069
65. Coleman R, Rathbone E, Marshall H, Wilson C, Brown J, Gossiel F, et al. Vitamin D, but not bone turnover markers, predict relapse in women with early breast cancer: an AZURE translational study. *Cancer Res.* (2012) 72:S6–4. doi: 10.1158/0008-5472.SABCS12-S6-4
66. Koopmans N, de Jong IJ, Breeuwsma AJ, van der Veer E. Serum bone turnover markers (PINP and ICTP) for the early detection of bone metastases in patients with prostate cancer: a longitudinal approach. *J Urol.* (2007) 178:849–53. doi: 10.1016/j.juro.2007.05.029
67. Lyubimova NV, Pashkov MV, Tyulyandin SA, Gol'dberg VE, Kushlinskii NE. Tartrate-resistant acid phosphatase as a marker of bone metastases in patients with breast cancer and prostate cancer. *Bull Exp Biol Med.* (2004) 138:77–9. doi: 10.1007/BF02694481
68. Salminen E, Ala-Houhala M, Korpela J, Varpula M, Tiitinen SL, Halleen JM, et al. Serum tartrate-resistant acid phosphatase 5b (TRACP 5b) as a marker of skeletal changes in prostate cancer. *Acta Oncol.* (2005) 44:742–7. doi: 10.1080/02841860500327586
69. Jung K, Lein M, Stephan C, Von Hösslin K, Semjonow A, Sinha P, et al. Comparison of 10 serum bone turnover markers in prostate carcinoma patients with bone metastatic spread: diagnostic and prognostic implications. *Int J Cancer.* (2004) 111:783–91. doi: 10.1002/ijc.20314
70. Jung K, Lein M, von Hosslin K, Brux B, Schnorr D, Loening SA, et al. Osteoprotegerin in serum as a novel marker of bone metastatic spread in prostate cancer. *Clin Chem.* (2001) 47:2061–3. doi: 10.1093/clinchem/47.11.2061
71. Ramankulov A, Lein M, Kristiansen G, Loening SA, Jung K. Plasma osteopontin in comparison with bone markers as indicator of bone metastasis and survival outcome in patients with prostate cancer. *Prostate.* (2007) 67:330–40. doi: 10.1002/pros.20540
72. Ramankulov A, Lein M, Kristiansen G, Meyer HA, Loening SA, Jung K. Elevated plasma osteopontin as marker for distant metastases and poor survival in patients with renal cell carcinoma. *J Cancer Res Clin Oncol.* (2007) 133:643–52. doi: 10.1007/s00432-007-0215-z
73. Tamiya M, Tokunaga S, Okada H, Suzuki H, Kobayashi M, Sasada S, et al. Prospective study of urinary and serum cross-linked N-telopeptide of type I collagen (NTx) for diagnosis of bone metastasis in patients with lung cancer. *Clin Lung Cancer.* (2013) 14:364–9. doi: 10.1016/j.cllc.2012.11.006
74. Brown JE, Cook RJ, Major P, Lipton A, Saad F, Smith M, et al. Bone turnover markers as predictors of skeletal complications in prostate cancer, lung cancer, and other solid tumors. *J Natl Cancer Inst.* (2005) 97:59–69. doi: 10.1093/jnci/dji002

75. Coleman RE, Major P, Lipton A, Brown JE, Lee KA, Smith M, et al. Predictive value of bone resorption and formation markers in cancer patients with bone metastases receiving the bisphosphonate zoledronic acid. *J Clin Oncol.* (2005) 23:4925–35. doi: 10.1200/JCO.2005.06.091
76. Costa L, Demers LM, Gouveia-Oliveira A, Schaller J, Costa EB, de Moura MC, et al. Prospective evaluation of the peptide-bound collagen type I cross-links N-telopeptide and C-telopeptide in predicting bone metastases status. *J Clin Oncol.* (2002) 20:850–6. doi: 10.1200/JCO.2002.20.3.850
77. Yao NS, Wu YY, Janckila AJ, Ku CH, Hsieh AT, Ho CL, et al. Serum tartrate-resistant acid phosphatase 5b (TRACP5b) activity as a biomarker for bone metastasis in non-small cell lung cancer patients. *Clin Chim Acta.* (2011) 412:181–5. doi: 10.1016/j.cca.2010.09.038
78. Mountzios G, Ramfidis V, Terpos E, Syrigos KN. Prognostic significance of bone markers in patients with lung cancer metastatic to the skeleton: a review of published data. *Clin Lung Cancer.* (2011) 12:341–9. doi: 10.1016/j.cllc.2011.03.032
79. Koizumi M, Yonese J, Fukui I, Ogata E. The serum level of the amino-terminal propeptide of type I procollagen is a sensitive marker for prostate cancer metastasis to bone. *BJU Int.* (2001) 87:348–51. doi: 10.1046/j.1464-410x.2001.00105.x
80. Zafeirakis AG, Papatheodorou GA, Limouris GS. Clinical and imaging correlations of bone turnover markers in prostate cancer patients with bone only metastases. *Nucl Med Commun.* (2010) 31:249–53. doi: 10.1097/MNM.0b013e328335a5ed
81. Yu M, Bardia A, Wittner BS, Stott SL, Smas ME, Ting DT, et al. Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. *Science.* (2013) 339:580–4. doi: 10.1126/science.1228522
82. Satelli A, Mitra A, Brownlee Z, Xia X, Bellister S, Overman MJ, et al. Epithelial-mesenchymal transitioned circulating tumor cells capture for detecting tumor progression. *Clin Cancer Res.* (2015) 21:899–906. doi: 10.1158/1078-0432.CCR-14-0894
83. Yokobori T, Iinuma H, Shimamura T, Imoto S, Sugimachi K, Ishii H, et al. Plastin3 is a novel marker for circulating tumor cells undergoing the epithelial-mesenchymal transition and is associated with colorectal cancer prognosis. *Cancer Res.* (2013) 73:2059–69. doi: 10.1158/0008-5472.CAN-12-0326

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MINI REVIEW
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Role of Altered Metabolic Microenvironment in Osteolytic Metastasis

*Kerstin Tiedemann[1,2], Osama Hussein[3] and Svetlana V. Komarova[1,2]**

[1] Faculty of Dentistry, McGill University, Montréal, QC, Canada, [2] Shriners Hospitals for Children – Canada, Montréal, QC, Canada, [3] Department of Surgery, Mansoura University Cancer Center, Mansoura, Egypt

Metastatic bone disease is generally incurable and leads to pathological fractures, pain, hypercalcemia, spinal cord compression and decreased mobility. The skeleton is the major site of bone metastases from solid cancers, including breast and prostate carcinoma. Bone metastasis is facilitated by activation of bone-resorbing osteoclasts, terminally differentiated multinucleated cells formed by fusion from monocytic precursors. Cancer cells are known to produce specific factors that stimulate osteoclast differentiation and function. Of interest, cancer cells are also known to alter their own bioenergetics increasing the use of glycolysis for their survival and function. Such change in energy utilization by cancer cells would result in altered levels of cell-permeable metabolites, including glucose, lactate, and pyruvate. Osteoclast resorption is energy-expensive, and we have previously demonstrated that during differentiation osteoclasts actively adapt to their bioenergetics microenvironment. We hypothesize that altered bioenergetics state of cancer cells will also modify the bioenergetics substrate availability for the tissue-resident bone cells, potentially creating a favorable milieu for pathological osteolysis. The goals of this review are to analyze how metastasizing cancer cells change the availability of energy substrates in bone microenvironment; and to assess how the altered bioenergetics may affect osteoclast differentiation and activity.

Keywords: bioenergetics, metabolism, osteoclast, bone microenvironment, cancer, osteolysis, metabolic sensors

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***Correspondence:**
Svetlana V. Komarova
svetlana.komarova@mcgill.ca

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INTRODUCTION

Bone is a preferred organ for metastasis from many tumors, including breast, prostate, and lung carcinomas (Hernandez et al., 2018). Establishment of metastatic bone lesions is facilitated by resident osteoclasts, cells that specialize in bone destruction. Molecular signatures that allow successful integration of cancer cells in the bone microenvironment have been extensively investigated (Olechnowicz and Edwards, 2014; Hiraga, 2019), however, none of the identified factors fully explains the success of tumors in thriving in the bone. In this mini-review, we will explore if tumor-mediated changes in bioenergetic environment may contribute to supporting osteoclast formation and function.

Cancer cells are different from their somatic counterparts in many factors, including their bioenergetics. Warburg effect, an increased use of anaerobic glycolysis by cancer cells, has re-gained

Abbreviations: AMPK, AMP-activated protein kinase; mTOR, Mammalian target of rapamycin; MCT, Monocarboxylate transporters; PGC-1β, Peroxisome proliferator–activated receptor-c coactivator 1β; RANKL, Receptor activator of nuclear factor kappa B-ligand; SLC, Solute carrier transporters.

much attention in the recent years (Lunt and Vander Heiden, 2011; Liberti and Locasale, 2016). The benefits of upregulating glycolysis for cancer cells are not fully understood, since oxidation of one molecule of glucose into pyruvate and 36 molecules of ATP per glucose are produced lactate during glycolysis generates 2 molecules of ATP, while 36 molecules of ATP per glucose are produced during oxidative phosphorylation. However, glycolysis is also important for biosynthesis of nucleotides, lipids and amino acids, all required for cellular proliferation (Lunt and Vander Heiden, 2011). Many metabolites involved in glycolysis and Krebs cycle are transported by the solute-carrier gene (SLC) family of membrane-bound transporters (Markovich and Murer, 2004). Glucose transporters that belong to 2A family of SLCs, represent a rate-limiting step in glycolysis and are known to be strongly dysregulated in cancer cells (Adekola et al., 2012). Lactate and pyruvate are transported by monocarboxylate transporters MCT1-4 that belong to the 16A family of SLCs, and MCT1 and MCT4 are upregulated in several cancers (Jones and Morris, 2016; Li et al., 2018). Importantly, intracellular and extracellular pools of lactate and pyruvate interchange relatively fast (Quek et al., 2016), therefore changes in intracellular metabolite levels lead to corresponding changes in the extracellular environment of cancer cells.

All cells adapt their energy metabolism to changing levels of energy demands, as well as availability of energy substrates. AMP-activated protein kinase (AMPK) is stimulated by an increase in AMP/ATP ratio due to cells inability to meet the current energy demand (Finley and Haigis, 2009). AMPK acts to decrease metabolic expenditure and increase energy production (Gwinn et al., 2008). Mammalian target of rapamycin (mTOR) generally acts downstream of AMPK. Two mTOR complexes, mTORC1 (with raptor and PRAS40) and mTORC2 (with rictor, mSIN1, and proctor) have distinct roles. While mTORC1 regulates protein synthesis (Foster and Toschi, 2009) and the SLC-mediated metabolite transport (Taylor, 2014), mTORC2 is linked to cytoskeletal dynamics and cell survival (Gaubitz et al., 2015). The metabolic sensors, AMPK and mTOR are critical players in cellular adaptation to a varying bioenergetics environment.

The goal of this review is to examine how changes in extracellular glycolytic metabolites due to the presence of actively proliferating cancer cells may alter osteoclast metabolic support, differentiation and function.

BIOENERGETICS REQUIREMENTS OF OSTEOCLASTS

To understand how osteoclasts can be affected by the metabolic substrates, we need to consider the normal bioenergetic requirements of these cells at different stages of their differentiation and function. Osteoclasts are multinucleated cells formed by fusion of monocytes. Mature osteoclasts attach to bone matrix, forming a sealing zone, where proton pumps lower the extracellular pH to dissolve hydroxyapatite, and proteolytic enzymes are secreted to digest the organic matrix (Stenbeck, 2002). Osteoclasts survive for ∼7–10 days, after which they die primarily by apoptosis (Akchurin et al., 2008; Kopesky et al., 2014). Osteoclast differentiation and function place significant and varied demands for energy required for migration of monocytes for cell fusion, phospholipid synthesis for cell membrane growth, protein synthesis to gain resorptive capacity, action of ion pumps and secretion of proteolytic enzymes. To provide this energy, monocytes increase glucose and oxygen consumption within 24–48 h of exposure to RANKL (Kim et al., 2007), up-regulate metabolic enzymes involved in energy production (Czupalla et al., 2005), and generate abundant large mitochondria (Dudley and Spiro, 1961; Lemma et al., 2016; **Figure 1**). Mitochondrial biogenesis stimulated by peroxisome proliferator–activated receptor-c coactivator 1β (PGC-1β) is a pre-requisite of successful osteoclastogenesis (Ishii et al., 2009; Wei et al., 2010; Zeng et al., 2015; Zhang et al., 2018). During resorption, osteoclast glucose transport increases 2-fold (Williams et al., 1997) and mitochondria locate near resorption surface (Kawahara et al., 2009). ATP levels markedly increase during osteoclastogenesis (Le Nihouannen et al., 2010). AMPK and mTOR are important for osteoclast differentiation and function. Osteoclastogenesis is associated with changes in AMPK isoform composition (Fong et al., 2013) and AMPK negatively regulates early stages of osteoclast differentiation (Lee et al., 2010; Shah et al., 2010; Kang et al., 2013). Signaling through mTOR is critical for osteoclast formation and survival (Glantschnig et al., 2003; Sugatani and Hruska, 2005; Hu et al., 2016; Dai et al., 2017), while osteoclast fusion and cytoplasmic growth depend on mTOR-mediated Akt signaling (Tiedemann et al., 2017). Importantly, nutrient availability during osteoclast differentiation was shown to significantly affect AMPK, mTORC1 and mTORC2 complexes (Fong et al., 2013; Tiedemann et al., 2017). Thus, it is conceivable that changes in metabolic substrate accessibility due to the presence of proliferating cancer cells may directly affect osteoclast differentiation and function.

POTENTIAL EFFECTS OF ALTERATIONS IN METABOLIC ENVIRONMENT ON OSTEOCLASTS

Glucose

Glucose, transported by glucose transporters 1 and 3 (Kim et al., 2007), is the most effective bioenergetics substrate for supporting bone resorption (Williams et al., 1997). In the absence of glucose, fatty acids, ketone bodies, and lactate can support bone resorption at 20–30% of the levels achievable with glucose (Williams et al., 1997). Nevertheless, the dose-dependence of glucose effects is complex. An increase from less than 1 mM to 5–10 mM glucose was demonstrated to stimulate osteoclastogenesis (Kim et al., 2007), resorption (Williams et al., 1997), and osteoclastogenic signaling through p38 mitogen-activated protein kinase (Larsen et al., 2002) and calcium/calmodulin-dependent kinase II (CaMK II) (Larsen et al., 2005). In a mouse model of type 2 diabetes, moderate hyperglycemia [∼10 mM circulating glucose (Fernandez et al., 2001)] was associated with increased osteoclastogenesis (Kawashima et al., 2009). In contrast, high glucose concentrations inhibit osteoclastogenesis

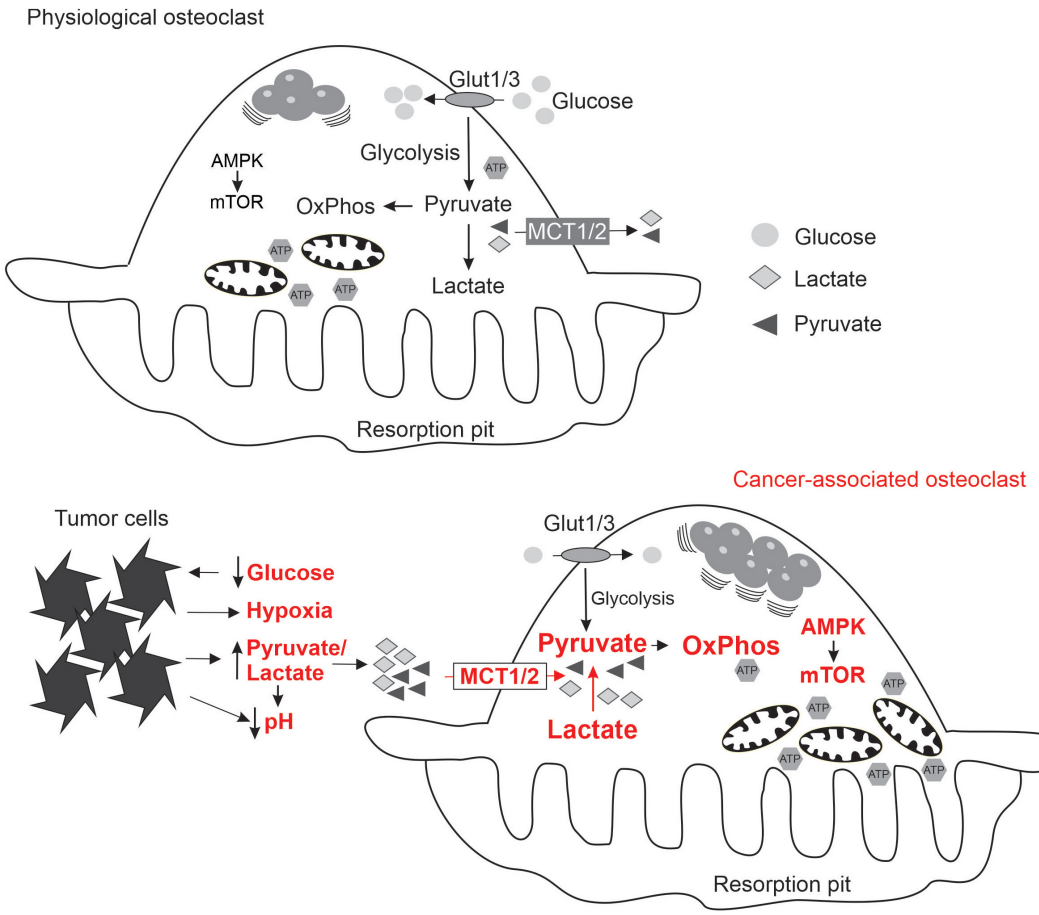

FIGURE 1 | Schematics summarizing the state of energy metabolism in osteoclasts formed under physiological conditions (top), and the adaptive changes in osteoclasts (indicated in red) exposed to the local microenvironment modified by metastasizing cancer cells (bottom).

(Kim et al., 2007; Wittrant et al., 2008), which could be explained by metabolic effects, such as decreased oxygen consumption at higher glucose level [similar to the Crabtree effect observed in yeasts (Pfeiffer and Morley, 2014)], as well as osmotic effects (Botolin and McCabe, 2006). In the environment of highly glycolytic cancer cells, the ambient glucose levels would likely decrease, reducing its availability for osteoclastogenesis. Thus, decrease in glucose is unlikely to contribute to osteoclastogenic effects of cancer cells.

Pyruvate

Several studies have investigated how pyruvate affects osteoclast formation. Addition of small amounts of pyruvate to media containing normal levels of glucose significantly increased osteoclastogenesis (Kim et al., 2007; Fong et al., 2013), resulting in formation of large osteoclasts that contained more nuclei per cell (Fong et al., 2013; Tiedemann et al., 2017). Of interest, only when added in relatively small amounts, between 1 and 2 mM (Fong et al., 2013; Tiedemann et al., 2017) and 5 mM (Kim et al., 2007), pyruvate was effective in promoting osteoclast formation. Addition of low pyruvate concentrations stimulated osteoclast mitochondrial activity, leading to a metabolic shift toward oxidative phosphorylation, and an increase in cellular [ATP] (Kim et al., 2007; Fong et al., 2013). Pyruvate caused an inhibition of AMPK and an activation of mTOR/raptor complex leading to facilitated protein synthesis and cytoplasmic growth (Fong et al., 2013; Tiedemann et al., 2017). MCT1, 2, and 4 for lactate and pyruvate are expressed by osteoclasts (Imai et al., 2019). MCT2 has the highest affinity for both pyruvate (Km ~0.1 mM) and lactate (Km ~0.7 mM), compared to MCT1 that has a Km value in millimolar range, and MCT4, affinity of which is even lower (Halestrap, 2012). Low concentration of MCT inhibitor or deletion of MCT1 were shown to potentiate osteoclastogenesis, while high concentration of MCT inhibitor or deletion of MCT2 prevented osteoclast formation (Imai et al., 2019). Another important issue with the interpretation of pyruvate effects was highlighted by Long and Halliwell (2009), who demonstrated that addition of pyruvate dramatically affects the media levels of hydrogen peroxide, which in turn affects osteoclastogenesis (Le Nihouannen et al., 2010). Nevertheless, no anti-oxidative effects were observed after addition of small amounts of pyruvate (Fong et al., 2013).

Increase in glycolysis due to Warburg effect in cancer cells can lead to increased production of pyruvate that can in turn be transported to the extracellular space (Doherty and Cleveland, 2013; Quek et al., 2016), and provide increased bioenergetic support for osteoclast formation.

Krebs Cycle Metabolites

Krebs cycle occurs in the mitochondria, however, several of its metabolites, including citrate, succinate, malate, oxaloacetate, fumarate, and α-ketoglutarate can be transported through the cell membrane by sodium-dependent SLC13 transporters (Markovich and Murer, 2004; Pajor, 2014). Citrate in particular gained a lot of interest, since its extracellular levels vary in diseases (Huang et al., 2020). Of particular interest is reported reduction in plasma citrate levels in prostate and lung cancers that readily metastasize to bone (Rocha et al., 2011; Dittrich et al., 2012), as well as in osteoporosis, in which citrate is also reduced in bone (major citrate reservoir) (Chen et al., 2018). Extracellular citrate affects osteoclastogenesis, however, contradictory outcomes were reported. Similar to pyruvate, 1–2 mM of sodium citrate was shown to enhance osteoclastogenesis (Fong et al., 2013). However, potassium citrate dose-dependently inhibited osteoclast formation at similar concentrations (Granchi et al., 2017). Importantly, osteoclast inhibition was also observed upon addition of potassium ion K^+ (KCl) (Yeon et al., 2015), suggesting that the effect of citrate may depend on media composition. Another potentially important link to Krebs cycle metabolites was proposed through glutamate metabolism. The glutamine transporter from SLC family 1a5 and glutaminase-1 converting glutamine to glutamate were shown to increase during osteoclastogenesis, leading authors to speculate that glutamate can be converted to α-ketoglutarate, which fuels energy metabolism (Indo et al., 2013). However, actively secretion of glutamate by osteoclasts was also demonstrated (Morimoto et al., 2006; Seidlitz et al., 2010). Thus, while glutamate likely plays an important role during osteoclastogenesis, it is difficult to conclude if its main action is relevant to energy metabolism. No information about other Krebs cycle intermediary is currently available. Thus, while the decreased citrate levels associated with cancer may affect osteoclastogenesis, the outcome of these interactions is uncertain and likely influenced by the localized cell microenvironment.

Mitochondria

The presence of highly proliferative cancer cells results in hypoxic microenvironment (Al Tameemi et al., 2019), which stimulates osteoclast differentiation and supports resorption (Arnett, 2010; Knowles, 2015). Hypoxic environment leads to a surprising improvement of mitochondrial function and ATP production in osteoclasts (Knowles, 2015), which may be due to reduction in proton leak and uncoupled respiration noted in mitochondria exposed to low oxygen tension (Gnaiger et al., 2000). Mitochondria activity is also linked to the production of reactive oxygen species (ROS) such as peroxide and superoxide (Knowles, 2015). ROS generate oxidative stress, which is counteracted by cellular glutathione (GSH) producing its oxidized form, glutathione disulfide (GSSG). Oxidative stress has a bimodal effect on osteoclasts: while moderate stress resulting in GSH/GSSG decrease is stimulatory for osteoclastogenesis, severe stress leading to glutathione depletion inhibits resorption and limits osteoclast lifespan (Kim et al., 2006; Le Nihouannen et al., 2010; Domazetovic et al., 2017). Cancer cells also actively modulate their oxidative microenvironment by secreting antioxidants, such as peroxiredoxin 4 (Rafiei et al., 2015; Tiedemann et al., 2019), suggesting tumor-associated oxidative stress may differ for tumor types and stages of their growth. Additionally, oxidative stress is also induced by chemotherapy, such as doxorubicin (Rana et al., 2013). Thus, hypoxia and potentially oxidative stress generated by cancer cells may provide a microenvironment that supports osteoclastogenesis.

pH and Lactate

Changes in pH are integral to the metabolic glucose processing. Anaerobic glycolysis results in acidification due to production of two molecules of lactic acid per each glucose (lactic acidosis), while complete mitochondrial oxidation of glucose generates six protons per glucose. Active metabolism of proliferating cancer cells is well recognized to produce acidic extracellular environment (Corbet and Feron, 2017). Acidification is also known to be a prerequisite of successful osteoclastogenesis (Arnett, 2010; Yuan et al., 2016; Arnett and Orriss, 2018). Osteoclasts sense extracellular acidosis through the G-protein coupled receptors, including ovarian cancer G-protein-coupled receptor 1 (OGR1) (Yang et al., 2006; Pereverzev et al., 2008; Li et al., 2009; Yuan et al., 2014) and T cell death-associated gene 8 (TDAG8) (Hikiji et al., 2014). In addition, osteoclasts express acid-sensitive ion channels (ASIC) (Jahr et al., 2005; Li et al., 2013). Acidosis was demonstrated to induce nuclear translocation of key osteoclastogenic transcription factor, nuclear factor of activated T cells 1c (NFATc1) (Komarova et al., 2005; Li et al., 2013) resulting in improved osteoclast formation (Granchi et al., 2017), resorptive activity (Komarova et al., 2005; Ahn et al., 2016), and survival (Pereverzev et al., 2008). Lactate was shown to be taken up by osteoclast precursors via MCT1 and to drive oxidative phosphorylation thereby facilitating bone resorption (Lemma et al., 2017). Thus, tumor-associated tissue acidosis and increased extracellular lactate can be expected to promote osteoclast differentiation and activity.

Metabolic Adaptation of Osteoclasts to Cancer Microenvironment

Metastasizing cancer cells generate unique bioenergetics microenvironment: while normal substrates, glucose and oxygen, are consumed by cancer cells, and therefore not available for osteoclasts, cancer cells generate alternative substrates such as pyruvate and lactate. In addition, acidic, hypoxic and potentially oxidative environment is uniquely supportive for osteoclastogenesis. To successfully perform in this altered microenvironment, osteoclasts need metabolic sensors to adapt their energy metabolism (**Figure 1**). We have shown that soluble factors produced by breast cancer

cells induce a change in osteoclast mTOR signaling (Hussein et al., 2012). Moreover, targeting mTOR with rapamycin in the mouse model of experimental bone metastases resulted in a significant attenuation of cancer-induced osteolysis (Hussein et al., 2012; Abdelaziz et al., 2014), but had minimal effect on osteoclasts in the cancer-free bones of the same animals (Abdelaziz et al., 2015). These findings suggest that metabolic sensors are central for osteoclast adaptation to the metastatic microenvironment, and may represent therapeutic targets reviewed in the following section.

EFFECT OF BIOENERGETICS TARGETING THERAPIES ON BONE METASTASIS

Therapeutics targeting metabolic sensors, such as metformin for AMPK and rapamycin for mTOR, have been successfully used for many years in a number of conditions including diabetes (Kezic et al., 2018) and organ transplantation (Augustine et al., 2007; Nguyen et al., 2019). In this section we attempted to review available evidence for the effectiveness of metformin and rapamycin and their analogs in preventing and/or controlling bone metastases.

Metformin

Metformin is an anti-diabetic drug that activates AMPK (Faubert et al., 2015). In cancer cells, loss of AMPK induced a typical Warburg effect in transformed and non-transformed cells (Faubert et al., 2013), and promoted unchecked mTORC1 activity (Inoki et al., 2003). Activation of AMPK has multiple anti-tumor effects (Schulten, 2018), particularly in colorectal and prostate cancer patients (Coyle et al., 2016). In bone, in addition to its role in osteoclastogenesis, AMPK reduced the expression of osteoclastogenic cytokine RANKL (Lee et al., 2010; Wang et al., 2013; Cuyàs et al., 2017). While reports of treatment of bone metastases with metformin are sparse (Wang et al., 2013), a reduction in growth of primary tumor and metastases was demonstrated in a model of castration-resistant prostatic carcinoma upon treatment with metformin and simvastatin (Babcook et al., 2014). Limited number of reports regarding the effectiveness of metformin can be explained by the study that demonstrated that metformin looses its ability to activate AMPK in hypoxic conditions, which are commonly associated with growing tumor (Garofalo et al., 2013).

Rapamycin and Its Analogs

In preclinical models of breast cancer bone metastases, rapamycin reduced osteolysis and bone pain, and improved animal survival (Hussein et al., 2012; Abdelaziz et al., 2014). Everolimus, a rapamycin analog more selective toward mTORC1 pathway, was also effective in preventing or treating experimental bone metastases from breast (Simone et al., 2015; Browne et al., 2017), prostate (Morgan et al., 2008), and lung (Yu et al., 2014) cancers. Several clinical trials evaluated the effectiveness of everolimus therapy in the treatment of hormone-receptor positive, Her2/Neu negative advanced breast cancer patients. A phase III, double-blind, randomized international BOLERO-2 trial compared the combination of anti-estrogen aromatase inhibitor exemestane with everolimus or placebo in postmenopausal women with advanced breast cancer. In addition to increasing progression-free survival (Yardley et al., 2013), everolimus markedly decreased levels of bone resorption biomarkers in patients with or without bone metastases (Gnant et al., 2013). RADAR clinical trial reported the effectiveness of everolimus in increasing the time to progression in a phase II double-blind, placebo-controlled, randomized discontinuation study in advanced breast cancer patients with bone metastases only (Maass et al., 2013). Thus, targeting mTOR appears promising in preclinical and clinical studies.

OVERALL CONCLUSION

The presence of cancer cells in the bone microenvironment likely results in local hypoglycemia and hypoxia. However, an increased glycolysis due to the Warburg effect in cancer cells may provide alternative metabolic substrates such as superfluous pyruvate and lactate. Adaptation of osteoclasts to such environment likely require the activity of metabolic sensors AMPK and mTOR. Importantly, osteoclasts are known to successfully adapt their mitochondrial function to conditions of hypoxia, which in osteoclasts stimulates ATP production, differentiation and function (Knowles, 2015). Acidification is another cancer-driven change in the microenvironment that is known to be specifically stimulatory for osteoclast formation and function (Arnett and Orriss, 2018). Thus, osteoclasts formed in the osteolytic tumor lesions are likely different from physiologically formed in their reliance on alternative metabolic substrates, adjusted activity of metabolic sensors, and unusual mitochondria function. Of interest, the combination of syrosingopine-mediated inhibition of MCT1 and 2 with metformin was recently demonstrated to result in synthetic lethality for cancer cells (Benjamin et al., 2018). We suggest that such drug combinations may target both cancer cells and cancer-supportive osteoclasts alleviating destructive and painful bone metastasis.

AUTHOR CONTRIBUTIONS

KT and SK conceived the study, researched, and summarized the preclinical studies. OH researched and summarized the clinical studies. All authors contributed to manuscript writing and approved the final version of the manuscript.

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REFERENCES

Abdelaziz, D. M., Stone, L. S., and Komarova, S. V. (2014). Osteolysis and pain due to experimental bone metastases are improved by treatment with rapamycin. *Breast Cancer Res. Treat.* 143, 227–237. doi: 10.1007/s10549-013-2799-0

Abdelaziz, D. M., Stone, L. S., and Komarova, S. V. (2015). Localized experimental bone metastasis drives osteolysis and sensory hypersensitivity at distant non-tumor-bearing sites. *Breast Cancer Res. Treat.* 153, 9–20. doi: 10.1007/s10549-015-3517-x

Adekola, K., Rosen, S. T., and Shanmugam, M. (2012). Glucose transporters in cancer metabolism. *Curr. Opin. Oncol* 24, 650–654. doi: 10.1097/CCO.0b013e328356da72

Ahn, H., Lee, K., Kim, J. M., Kwon, S. H., Lee, S. H., Lee, S. Y., et al. (2016). Accelerated lactate dehydrogenase activity potentiates osteoclastogenesis via NFATc1 signaling. *PLoS One* 11:e0153886. doi: 10.1371/journal.pone.0153886

Akchurin, T., Aissiou, T., Kemeny, N., Prosk, E., Nigam, N., and Komarova, S. V. (2008). Complex dynamics of osteoclast formation and death in long-term cultures. *PLoS One* 3:e2104. doi: 10.1371/journal.pone.0002104

Al Tameemi, W., Dale, T. P., Al-Jumaily, R. M. K., and Forsyth, N. R. (2019). Hypoxia-modified cancer cell metabolism. *Front. Cell Dev. Biol.* 7:4. doi: 10.3389/fcell.2019.00004

Arnett, T. R. (2010). Acidosis, hypoxia and bone. *Arch. Biochem. Biophys.* 503, 103–109. doi: 10.1016/j.abb.2010.07.021

Arnett, T. R., and Orriss, I. R. (2018). Metabolic properties of the osteoclast. *Bone* 115, 25–30. doi: 10.1016/j.bone.2017.12.021

Augustine, J. J., Bodziak, K. A., and Hricik, D. E. (2007). Use of sirolimus in solid organ transplantation. *Drugs* 67, 369–391. doi: 10.2165/00003495-200767030-00004

Babcook, M. A., Shukla, S., Fu, P., Vazquez, E. J., Puchowicz, M. A., Molter, J. P., et al. (2014). Synergistic simvastatin and metformin combination chemotherapy for osseous metastatic castration-resistant prostate cancer. *Mol. Cancer Ther.* 13, 2288–2302. doi: 10.1158/1535-7163.mct-14-0451

Benjamin, D., Robay, D., Hindupur, S. K., Pohlmann, J., Colombi, M., El-Shemerly, M. Y., et al. (2018). Dual inhibition of the lactate transporters MCT1 and MCT4 is synthetic lethal with metformin due to NAD+ depletion in cancer cells. *Cell Rep.* 25, 3047.e4–3058.e4. doi: 10.1016/j.celrep.2018.11.043

Botolin, S., and McCabe, L. R. (2006). Chronic hyperglycemia modulates osteoblast gene expression through osmotic and non-osmotic pathways. *J. Cell. Biochem.* 99, 411–424. doi: 10.1002/jcb.20842

Browne, A. J., Kubasch, M. L., Gobel, A., Hadji, P., Chen, D., Rauner, M., et al. (2017). Concurrent antitumor and bone-protective effects of everolimus in osteotropic breast cancer. *Breast Cancer Res.* 19:92. doi: 10.1186/s13058-017-0885-7

Chen, H., Wang, Y., Dai, H., Tian, X., Cui, Z. K., Chen, Z., et al. (2018). Bone and plasma citrate is reduced in osteoporosis. *Bone* 114, 189–197. doi: 10.1016/j.bone.2018.06.014

Corbet, C., and Feron, O. (2017). Tumour acidosis: from the passenger to the driver's seat. *Nat. Rev. Cancer* 17, 577–593. doi: 10.1038/nrc.2017.77

Coyle, C., Cafferty, F. H., Vale, C., and Langley, R. E. (2016). Metformin as an adjuvant treatment for cancer: a systematic review and meta-analysis. *Ann. Oncol.* 27, 2184–2195. doi: 10.1093/annonc/mdw410

Cuyàs, E., Martin-Castillo, B., Bosch-Barrera, J., and Menendez, J. A. (2017). Metformin inhibits RANKL and sensitizes cancer stem cells to denosumab. *Cell Cycle* 16, 1022–1028. doi: 10.1080/15384101.2017.1310353

Czupalla, C., Mansukoski, H., Pursche, T., Krause, E., and Hoflack, B. (2005). Comparative study of protein and mRNA expression during osteoclastogenesis. *Proteomics* 5, 3868–3875. doi: 10.1002/pmic.200402059

Dai, Q., Xie, F., Han, Y., Ma, X., Zhou, S., Jiang, L., et al. (2017). Inactivation of regulatory-associated Protein of mTOR (Raptor)/mammalian target of rapamycin complex 1 (mTORC1) signaling in osteoclasts increases bone mass by inhibiting osteoclast differentiation in mice. *J. Biol. Chem.* 292, 196–204. doi: 10.1074/jbc.M116.764761

Dittrich, R., Kurth, J., Decelle, E. A., DeFeo, E. M., Taupitz, M., Wu, S., et al. (2012). Assessing prostate cancer growth with citrate measured by intact tissue proton magnetic resonance spectroscopy. *Prostate Cancer Prostatic. Dis.* 15, 278–282. doi: 10.1038/pcan.2011.70

Doherty, J. R., and Cleveland, J. L. (2013). Targeting lactate metabolism for cancer therapeutics. *J. Clin. Invest.* 123, 3685–3692. doi: 10.1172/jci69741

Domazetovic, V., Marcucci, G., Iantomasi, T., Brandi, M. L., and Vincenzini, M. T. (2017). Oxidative stress in bone remodeling: role of antioxidants. *Clin. Cases Miner. Bone Metab.* 14, 209–216. doi: 10.11138/ccmbm/2017.14.1.209

Dudley, H. R., and Spiro, D. (1961). The fine structure of bone cells. *J. Biophys. Biochem. Cytol* 11, 627–649. doi: 10.1083/jcb.11.3.627

Faubert, B., Boily, G., Izreig, S., Griss, T., Samborska, B., Dong, Z., et al. (2013). AMPK is a negative regulator of the warburg effect and suppresses tumor growth in vivo. *Cell Metab.* 17, 113–124. doi: 10.1016/j.cmet.2012.12.001

Faubert, B., Vincent, E. E., Poffenberger, M. C., and Jones, R. G. (2015). The AMP-activated protein kinase (AMPK) and cancer: many faces of a metabolic regulator. *Cancer Metab.* 356(2, Pt A), 165–170. doi: 10.1016/j.canlet.2014.01.018

Fernandez, A. M., Kim, J. K., Yakar, S., Dupont, J., Hernandez-Sanchez, C., Castle, A. L., et al. (2001). Functional inactivation of the IGF-I and insulin receptors in skeletal muscle causes type 2 diabetes. *Genes Dev.* 15, 1926–1934. doi: 10.1101/gad.908001

Finley, L. W., and Haigis, M. C. (2009). The coordination of nuclear and mitochondrial communication during aging and calorie restriction. *Ageing Res. Rev.* 8, 173–188. doi: 10.1016/j.arr.2009.03.003

Fong, J. E., Le Nihouannen, D., Tiedemann, K., Sadvakassova, G., Barralet, J. E., and Komarova, S. V. (2013). Moderate excess of pyruvate augments osteoclastogenesis. *Biol. Open* 2, 387–395. doi: 10.1242/bio.20133269

Foster, D. A., and Toschi, A. (2009). Targeting mTOR with rapamycin: one dose does not fit all. *Cell Cycle* 8, 1026–1029. doi: 10.4161/cc.8.7.8044

Garofalo, C., Capristo, M., Manara, M. C., Mancarella, C., Landuzzi, L., Belfiore, A., et al. (2013). Metformin as an adjuvant drug against pediatric sarcomas: hypoxia limits therapeutic effects of the drug. *PLoS One* 8:e83832. doi: 10.1371/journal.pone.0083832

Gaubitz, C., Oliveira, T. M., Prouteau, M., Leitner, A., Karuppasamy, M., Konstantinidou, G., et al. (2015). Molecular basis of the rapamycin insensitivity of target of rapamycin complex 2. *Mol. Cell.* 58, 977–988. doi: 10.1016/j.molcel.2015.04.031

Glantschnig, H., Fisher, J. E., Wesolowski, G., Rodan, G. A., and Reszka, A. A. (2003). M-CSF, TNFalpha and RANK ligand promote osteoclast survival by signaling through mTOR/S6 kinase. *Cell Death. Differ.* 10, 1165–1177. doi: 10.1038/sj.cdd.4401285

Gnaiger, E., Mendez, G., and Hand, S. C. (2000). High phosphorylation efficiency and depression of uncoupled respiration in mitochondria under hypoxia. *Proc. Natl. Acad. Sci. U.S.A.* 97, 11080–11085. doi: 10.1073/pnas.97.20.11080

Gnant, M., Baselga, J., Rugo, H. S., Noguchi, S., Burris, H. A., Piccart, M., et al. (2013). Effect of everolimus on bone marker levels and progressive disease in bone in BOLERO-2. *J. Natl. Cancer Inst.* 105, 654–663. doi: 10.1093/jnci/djt026

Granchi, D., Torreggiani, E., Massa, A., Caudarella, R., Di Pompo, G., and Baldini, N. (2017). Potassium citrate prevents increased osteoclastogenesis resulting from acidic conditions: implication for the treatment of postmenopausal bone loss. *PLoS One* 12:e0181230. doi: 10.1371/journal.pone.0181230

Gwinn, D. M., Shackelford, D. B., Egan, D. F., Mihaylova, M. M., Mery, A., Vasquez, D. S., et al. (2008). AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Mol. Cell.* 30, 214–226. doi: 10.1016/j.molcel.2008.03.003

Halestrap, A. P. (2012). The monocarboxylate transporter family–Structure and functional characterization. *IUBMB Life* 64, 1–9. doi: 10.1002/iub.573

Hernandez, R. K., Wade, S. W., Reich, A., Pirolli, M., Liede, A., and Lyman, G. H. (2018). Incidence of bone metastases in patients with solid tumors: analysis of oncology electronic medical records in the United States. *BMC Cancer* 18:44. doi: 10.1186/s12885-017-3922-0

Hikiji, H., Endo, D., Horie, K., Harayama, T., Akahoshi, N., Igarashi, H., et al. (2014). TDAG8 activation inhibits osteoclastic bone resorption. *FASEB J.* 28, 871–879. doi: 10.1096/fj.13-233106

Hiraga, T. (2019). Bone metastasis: interaction between cancer cells and bone microenvironment. *J. Oral. Biosci.* 61, 95–98. doi: 10.1016/j.job.2019.02.002

Hu, Y., Carraro-Lacroix, L. R., Wang, A., Owen, C., Bajenova, E., Corey, P. N., et al. (2016). Lysosomal pH plays a key role in regulation of mtor activity in osteoclasts. *J. Cell. Biochem.* 117, 413–425. doi: 10.1002/jcb.25287

Huang, L., Wang, C., Xu, H., and Peng, G. (2020). Targeting citrate as a novel therapeutic strategy in cancer treatment. *Biochim. Biophys. Acta Rev. Cancer* 1873:188332. doi: 10.1016/j.bbcan.2019.188332

Hussein, O., Tiedemann, K., Murshed, M., and Komarova, S. V. (2012). Rapamycin inhibits osteolysis and improves survival in a model of experimental

bone metastases. *Cancer Lett.* 314, 176–184. doi: 10.1016/j.canlet.2011.09.026

Imai, H., Yoshimura, K., Miyamoto, Y., Sasa, K., Sugano, M., Chatani, M., et al. (2019). Roles of monocarboxylate transporter subtypes in promotion and suppression of osteoclast differentiation and survival on bone. *Sci. Rep.* 9:15608. doi: 10.1038/s41598-019-52128-2

Indo, Y., Takeshita, S., Ishii, K. A., Hoshii, T., Aburatani, H., Hirao, A., et al. (2013). Metabolic regulation of osteoclast differentiation and function. *J. Bone. Miner. Res.* 28, 2392–2399. doi: 10.1002/jbmr.1976

Inoki, K., Zhu, T., and Guan, K.-L. (2003). TSC2 mediates cellular energy response to control cell growth and survival. *Cell* 115, 577–590.

Ishii, K. A., Fumoto, T., Iwai, K., Takeshita, S., Ito, M., Shimohata, N., et al. (2009). Coordination of PGC-1beta and iron uptake in mitochondrial biogenesis and osteoclast activation. *Nat. Med.* 15, 259–266. doi: 10.1038/nm.1910

Jahr, H., van Driel, M., van Osch, G. J., Weinans, H., and van Leeuwen, J. P. (2005). Identification of acid-sensing ion channels in bone. *Biochem. Biophys. Res. Commun.* 337, 349–354. doi: 10.1016/j.bbrc.2005.09.054

Jones, R. S., and Morris, M. E. (2016). Monocarboxylate transporters: therapeutic targets and prognostic factors in disease. *Clin. Pharmacol. Ther* 100, 454–463. doi: 10.1002/cpt.418

Kang, H., Viollet, B., and Wu, D. (2013). Genetic deletion of catalytic subunits of AMP-activated protein kinase increases osteoclasts and reduces bone mass in young adult mice. *J. Biol. Chem.* 288, 12187–12196. doi: 10.1074/jbc.M112.430389

Kawahara, I., Koide, M., Tadokoro, O., Udagawa, N., Nakamura, H., Takahashi, N., et al. (2009). The relationship between calcium accumulation in osteoclast mitochondrial granules and bone resorption. *Bone* 45, 980–986. doi: 10.1016/j.bone.2009.07.010

Kawashima, Y., Fritton, J. C., Yakar, S., Epstein, S., Schaffler, M. B., Jepsen, K. J., et al. (2009). Type 2 diabetic mice demonstrate slender long bones with increased fragility secondary to increased osteoclastogenesis. *Bone* 44, 648–655. doi: 10.1016/j.bone.2008.12.012

Kezic, A., Popovic, L., and Lalic, K. (2018). mTOR inhibitor therapy and metabolic consequences: where do we stand? *Oxid Med. Cell Longev* 2018:2640342. doi: 10.1155/2018/2640342

Kim, H., Kim, I. Y., Lee, S. Y., and Jeong, D. (2006). Bimodal actions of reactive oxygen species in the differentiation and bone-resorbing functions of osteoclasts. *FEBS Lett.* 580, 5661–5665. doi: 10.1016/j.febslet.2006.09.015

Kim, J. M., Jeong, D., Kang, H. K., Jung, S. Y., Kang, S. S., and Min, B. M. (2007). Osteoclast precursors display dynamic metabolic shifts toward accelerated glucose metabolism at an early stage of RANKL-stimulated osteoclast differentiation. *Cell. Physiol. Biochem.* 20, 935–946.

Knowles, H. J. (2015). Hypoxic regulation of osteoclast differentiation and bone resorption activity. *Hypoxia* 3, 73–82. doi: 10.2147/hp.s95960

Komarova, S. V., Pereverzev, A., Shum, J. W., Sims, S. M., and Dixon, S. J. (2005). Convergent signaling by acidosis and receptor activator of NF-kappaB ligand (RANKL) on the calcium/calcineurin/NFAT pathway in osteoclasts. *Proc. Natl. Acad. Sci. U.S.A.* 102, 2643–2648. doi: 10.1073/pnas.0406874102

Kopesky, P., Tiedemann, K., Alkekhia, D., Zechner, C., Millard, B., Schoeberl, B., et al. (2014). Autocrine signaling is a key regulatory element during osteoclastogenesis. *Biol. Open* 3, 767–776. doi: 10.1242/bio.20148128

Larsen, K. I., Falany, M. L., Ponomareva, L. V., Wang, W., and Williams, J. P. (2002). Glucose-dependent regulation of osteoclast H(+)-ATPase expression: potential role of p38 MAP-kinase. *J. Cell. Biochem.* 87, 75–84. doi: 10.1002/jcb.10252

Larsen, K. I., Falany, M., Wang, W., and Williams, J. P. (2005). Glucose is a key metabolic regulator of osteoclasts; glucose stimulated increases in ATP/ADP ratio and calmodulin kinase II activity. *Biochem. Cell Biol.* 83, 667–673. doi: 10.1139/o05-136

Le Nihouannen, D., Barralet, J. E., Fong, J. E., and Komarova, S. V. (2010). Ascorbic acid accelerates osteoclast formation and death. *Bone* 46, 1336–1343. doi: 10.1016/j.bone.2009.11.021

Lee, Y. S., Kim, Y. S., Lee, S. Y., Kim, G. H., Kim, B. J., Lee, S. H., et al. (2010). AMP kinase acts as a negative regulator of RANKL in the differentiation of osteoclasts. *Bone* 47, 926–937. doi: 10.1016/j.bone.2010.08.001

Lemma, S., Di Pompo, G., Porporato, P. E., Sboarina, M., Russell, S., Gillies, R. J., et al. (2017). MDA-MB-231 breast cancer cells fuel osteoclast metabolism and activity: a new rationale for the pathogenesis of osteolytic bone metastases. *Biochim. Biophys. Acta Mol. Basis Dis.* 1863, 3254–3264. doi: 10.1016/j.bbadis.2017.08.030

Lemma, S., Sboarina, M., Porporato, P. E., Zini, N., Sonveaux, P., Di Pompo, G., et al. (2016). Energy metabolism in osteoclast formation and activity. *Int. J. Biochem. Cell Biol.* 79, 168–180. doi: 10.1016/j.biocel.2016.08.034

Li, H., Wang, D., Singh, L. S., Berk, M., Tan, H., Zhao, Z., et al. (2009). Abnormalities in osteoclastogenesis and decreased tumorigenesis in mice deficient for ovarian cancer G protein-coupled receptor 1. *PLoS One* 4:e5705. doi: 10.1371/journal.pone.0005705

Li, X., Xu, R. S., Jiang, D. L., He, X. L., Jin, C., Lu, W. G., et al. (2013). Acid-sensing ion channel 1a is involved in acid-induced osteoclastogenesis by regulating activation of the transcription factor NFATc1. *FEBS Lett.* 587, 3236–3242. doi: 10.1016/j.febslet.2013.08.017

Li, Z., Wu, Q., Sun, S., Wu, J., Li, J., Zhang, Y., et al. (2018). Monocarboxylate transporters in breast cancer and adipose tissue are novel biomarkers and potential therapeutic targets. *Biochem. Biophys. Res. Commun.* 501, 962–967. doi: 10.1016/j.bbrc.2018.05.091

Liberti, M. V., and Locasale, J. W. (2016). The warburg effect: how does it benefit cancer cells? *Trends Biochem. Sci.* 41, 211–218. doi: 10.1016/j.tibs.2015.12.001

Long, L. H., and Halliwell, B. (2009). Artefacts in cell culture: pyruvate as a scavenger of hydrogen peroxide generated by ascorbate or epigallocatechin gallate in cell culture media. *Biochem. Biophys. Res. Commun.* 388, 700–704. doi: 10.1016/j.bbrc.2009.08.069

Lunt, S. Y., and Vander Heiden, M. G. (2011). Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annu. Rev. Cell Dev. Biol.* 27, 441–464. doi: 10.1146/annurev-cellbio-092910-154237

Maass, N., Harbeck, N., Mundhenke, C., Lerchenmuller, C., Barinoff, J., Luck, H. J., et al. (2013). Everolimus as treatment for breast cancer patients with bone metastases only: results of the phase II RADAR study. *J. Cancer Res. Clin. Oncol.* 139, 2047–2056. doi: 10.1007/s00432-013-1518-x

Markovich, D., and Murer, H. (2004). The SLC13 gene family of sodium sulphate/carboxylate cotransporters. *Pflugers. Arch.* 447, 594–602. doi: 10.1007/s00424-003-1128-6

Morgan, T. M., Pitts, T. E., Gross, T. S., Poliachik, S. L., Vessella, R. L., and Corey, E. (2008). RAD001 (Everolimus) inhibits growth of prostate cancer in the bone and the inhibitory effects are increased by combination with docetaxel and zoledronic acid. *Prostate* 68, 861–871. doi: 10.1002/pros.20752

Morimoto, R., Uehara, S., Yatsushiro, S., Juge, N., Hua, Z., Senoh, S., et al. (2006). Secretion of L-glutamate from osteoclasts through transcytosis. *EMBO J.* 25, 4175–4186. doi: 10.1038/sj.emboj.7601317

Nguyen, L. S., Vautier, M., Allenbach, Y., Zahr, N., Benveniste, O., Funck-Brentano, C., et al. (2019). Sirolimus and mTOR inhibitors: a review of side effects and specific management in solid organ transplantation. *Drug Saf.* 42, 813–825. doi: 10.1007/s40264-019-00810-9

Olechnowicz, S. W., and Edwards, C. M. (2014). Contributions of the host microenvironment to cancer-induced bone disease. *Cancer Res.* 74, 1625–1631. doi: 10.1158/0008-5472.Can-13-2645

Pajor, A. M. (2014). Sodium-coupled dicarboxylate and citrate transporters from the SLC13 family. *Pflugers. Arch.* 466, 119–130. doi: 10.1007/s00424-013-1369-y

Pereverzev, A., Komarova, S. V., Korcok, J., Armstrong, S., Tremblay, G. B., Dixon, S. J., et al. (2008). Extracellular acidification enhances osteoclast survival through an NFAT-independent, protein kinase C-dependent pathway. *Bone* 42, 150–161. doi: 10.1016/j.bone.2007.08.044

Pfeiffer, T., and Morley, A. (2014). An evolutionary perspective on the crabtree effect. *Front. Mol. Biosci.* 1:17. doi: 10.3389/fmolb.2014.00017

Quek, L. E., Liu, M., Joshi, S., and Turner, N. (2016). Fast exchange fluxes around the pyruvate node: a leaky cell model to explain the gain and loss of unlabelled and labelled metabolites in a tracer experiment. *Cancer Metab.* 4:3. doi: 10.1186/s40170-016-0153-9

Rafiei, S., Tiedemann, K., Tabaries, S., Siegel, P. M., and Komarova, S. V. (2015). Peroxiredoxin 4: a novel secreted mediator of cancer induced osteoclastogenesis. *Cancer Lett.* 361, 262–270. doi: 10.1016/j.canlet.2015.03.012

Rana, T., Chakrabarti, A., Freeman, M., and Biswas, S. (2013). Doxorubicin-mediated bone loss in breast cancer bone metastases is driven by an interplay between oxidative stress and induction of TGFbeta. *PLoS One* 8:e78043. doi: 10.1371/journal.pone.0078043

Rocha, C. M., Carrola, J., Barros, A. S., Gil, A. M., Goodfellow, B. J., Carreira, I. M., et al. (2011). Metabolic signatures of lung cancer in biofluids: NMR-based metabonomics of blood plasma. *J. Proteome Res.* 10, 4314–4324. doi: 10.1021/pr200550p

Schulten, H. J. (2018). Pleiotropic effects of metformin on cancer. *Int. J. Mol. Sci.* 19:2850. doi: 10.3390/ijms19102850

Seidlitz, E. P., Sharma, M. K., and Singh, G. (2010). Extracellular glutamate alters mature osteoclast and osteoblast functions. *Can. J. Physiol. Pharmacol.* 88, 929–936. doi: 10.1139/y10-070

Shah, M., Kola, B., Bataveljic, A., Arnett, T. R., Viollet, B., Saxon, L., et al. (2010). AMP-activated protein kinase (AMPK) activation regulates in vitro bone formation and bone mass. *Bone* 47, 309–319. doi: 10.1016/j.bone.2010.04.596

Simone, V., Ciavarella, S., Brunetti, O., Savonarola, A., Cives, M., Tucci, M., et al. (2015). Everolimus restrains the paracrine pro-osteoclast activity of breast cancer cells. *BMC Cancer* 15:692. doi: 10.1186/s12885-015-1717-8

Stenbeck, G. (2002). Formation and function of the ruffled border in osteoclasts. *Semin. Cell Dev. Biol.* 13, 285–292. doi: 10.1016/s1084952102000587

Sugatani, T., and Hruska, K. A. (2005). Akt1/Akt2 and mammalian target of rapamycin/Bim play critical roles in osteoclast differentiation and survival, respectively, whereas Akt is dispensable for cell survival in isolated osteoclast precursors. *J. Biol. Chem.* 280, 3583–3589. doi: 10.1074/jbc.M410480200

Taylor, P. M. (2014). Role of amino acid transporters in amino acid sensing. *Am. J. Clin. Nutr.* 99, 223s–230s. doi: 10.3945/ajcn.113.070086

Tiedemann, K., Le Nihouannen, D., Fong, J. E., Hussein, O., Barralet, J. E., and Komarova, S. V. (2017). Regulation of osteoclast growth and fusion by mTOR/raptor and mTOR/rictor/Akt. *Front. Cell Dev. Biol.* 5:54. doi: 10.3389/fcell.2017.00054

Tiedemann, K., Sadvakassova, G., Mikolajewicz, N., Juhas, M., Sabirova, Z., Tabaries, S., et al. (2019). Exosomal release of L-plastin by breast cancer cells facilitates metastatic bone osteolysis. *Transl. Oncol.* 12, 462–474. doi: 10.1016/j.tranon.2018.11.014

Wang, J., Chen, T. Y., Qin, S., Duan, Y., and Wang, G. (2013). Inhibitory effect of metformin on bone metastasis of cancer via OPG/RANKL/RANK system. *Med. Hypotheses* 81, 805–806. doi: 10.1016/j.mehy.2013.08.032

Wei, W., Wang, X., Yang, M., Smith, L. C., Dechow, P. C., Sonoda, J., et al. (2010). PGC1beta mediates PPARgamma activation of osteoclastogenesis and rosiglitazone-induced bone loss. *Cell Metab.* 11, 503–516. doi: 10.1016/j.cmet.2010.04.015

Williams, J. P., Blair, H. C., McDonald, J. M., McKenna, M. A., Jordan, S. E., Williford, J., et al. (1997). Regulation of osteoclastic bone resorption by glucose. *Biochem. Biophys. Res. Commun.* 235, 646–651. doi: 10.1006/bbrc.1997.6795

Wittrant, Y., Gorin, Y., Woodruff, K., Horn, D., Abboud, H. E., Mohan, S., et al. (2008). High d(+)glucose concentration inhibits RANKL-induced osteoclastogenesis. *Bone* 42, 1122–1130. doi: 10.1016/j.bone.2008.02.006

Yang, M., Mailhot, G., Birnbaum, M. J., MacKay, C. A., Mason-Savas, A., and Odgren, P. R. (2006). Expression of and role for ovarian cancer G-protein-coupled receptor 1 (OGR1) during osteoclastogenesis. *J. Biol. Chem.* 281, 23598–23605. doi: 10.1074/jbc.M602191200

Yardley, D. A., Noguchi, S., Pritchard, K. I., Burris, H. A., Baselga, J., Gnant, M., et al. (2013). Everolimus plus exemestane in postmenopausal patients with HR(+) breast cancer: BOLERO-$_2$ final progression-free survival analysis. *Adv. Ther.* 30, 870–884. doi: 10.1007/s12325-013-0060-1

Yeon, J. T., Kim, K. J., Chun, S. W., Lee, H. I., Lim, J. Y., Son, Y. J., et al. (2015). KCNK1 inhibits osteoclastogenesis by blocking the Ca2+ oscillation and JNK-NFATc1 signaling axis. *J. Cell Sci.* 128, 3411–3419. doi: 10.1242/jcs.170738

Yu, Y., Song, Z., Yang, S., Yang, X., Zhang, J., and Lu, S. (2014). Everolimus and zoledronic acid–a potential synergistic treatment for lung adenocarcinoma bone metastasis. *Acta Biochim. Biophys. Sin.* 46, 792–801. doi: 10.1093/abbs/gmu069

Yuan, F. L., Xu, M. H., Li, X., Xinlong, H., Fang, W., and Dong, J. (2016). The roles of acidosis in osteoclast biology. *Front. Physiol.* 7:222. doi: 10.3389/fphys.2016.00222

Yuan, F. L., Zhao, M. D., Jiang, L. B., Wang, H. R., Cao, L., Zhou, X. G., et al. (2014). Molecular actions of ovarian cancer G protein-coupled receptor 1 caused by extracellular acidification in bone. *Int. J. Mol. Sci* 15, 22365–22373. doi: 10.3390/ijms151222365

Zeng, R., Faccio, R., and Novack, D. V. (2015). Alternative NF-kappaB regulates RANKL-induced osteoclast differentiation and mitochondrial biogenesis via independent mechanisms. *J. Bone Mineral Res.* 30, 2287–2299. doi: 10.1002/jbmr.2584

Zhang, Y., Rohatgi, N., Veis, D. J., Schilling, J., Teitelbaum, S. L., and Zou, W. (2018). PGC1beta organizes the osteoclast cytoskeleton by mitochondrial biogenesis and activation. *J. Bone Mineral Res.* 33, 1114–1125. doi: 10.1002/jbmr.3398

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REVIEW
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Osteoclast Signal Transduction During Bone Metastasis Formation

Dávid S. Győri and Attila Mócsai*

Department of Physiology, Faculty of Medicine, Semmelweis University, Budapest, Hungary

Osteoclasts are myeloid lineage-derived bone-resorbing cells of hematopoietic origin. They differentiate from myeloid precursors through a complex regulation process where the differentiation of preosteoclasts is followed by intercellular fusion to generate large multinucleated cells. Under physiological conditions, osteoclastogenesis is primarily directed by interactions between CSF-1R and macrophage colony-stimulating factor (M-CSF, CSF-1), receptor activator of nuclear factor NF-κB (RANK) and RANK ligand (RANKL), as well as adhesion receptors (e.g., integrins) and their ligands. Osteoclasts play a central role in physiological and pathological bone resorption and are also required for excessive bone loss during osteoporosis, inflammatory bone and joint diseases (such as rheumatoid arthritis) and cancer cell-induced osteolysis. Due to the major role of osteoclasts in these diseases the better understanding of their intracellular signaling pathways can lead to the identification of potential novel therapeutic targets. Non-receptor tyrosine kinases and lipid kinases play major roles in osteoclasts and small-molecule kinase inhibitors are emerging new therapeutics in diseases with pathological bone loss. During the last few years, we and others have shown that certain lipid (such as phosphoinositide 3-kinases PI3Kβ and PI3Kδ) and tyrosine (Src−family and Syk) kinases play a critical role in osteoclast differentiation and function in humans and mice. Some of these signaling pathways shows similarity to immunoreceptor-like receptor signaling and involves important other enzymes (e.g., PLCγ2) and adapter proteins (such as the ITAM−bearing adapters DAP12 and the Fc-receptor γ-chain). Here, we review recently identified osteoclast signaling pathways and their role in osteoclast differentiation and function as well as pathological bone loss associated with osteolytic tumors of the bone. A better understanding of osteoclast signaling may facilitate the design of novel and more efficient therapies for pathological bone resorption and osteolytic skeletal metastasis formation.

Keywords: osteoclast (OC), signaling/signaling pathways, tumor, bone metastases (BM), osteolysis

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Teun J. De Vries, VU University Amsterdam, Netherlands
Joseph Anthony Lorenzo, UCONN Health, United States
Deborah Veis, Washington University School of Medicine in St. Louis, United States

****Correspondence:***
Dávid S. Győri
gyori.david@med.semmelweis-univ.hu

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DEVELOPMENT AND FUNCTION OF OSTEOCLASTS AND THEIR ROLE IN PATHOLOGICAL BONE LOSS

Bone tissue plays a crucial role in structural support and movement of the body as well as it stores minerals. It also hosts the bone marrow, which is the major site of postnatal hematopoiesis (Zaidi, 2007). Bone matrix is an essential component of the bone and it is built up from inorganic salts and organic matrix. Besides providing structural support, bone matrix also stores a wide range of growth factors capable of regulating normal bone homeostasis. Bone microenvironment contains a wide repertoire of cellular elements: hematopoietic and mesenchymal stem cells, chondrocytes, fibroblasts, adipocytes, endothelial and nerve cells as well as the bone cells themselves

(Arron and Choi, 2000). The most well-known of these latter ones are bone-resorbing osteoclasts, bone-forming osteoblasts and osteocytes regulating the bone remodeling process (Bonewald, 2011).

Osteoclasts are derived from myeloid precursors, which express several cytokine receptors. Osteoclast differentiation is mainly governed by receptor activator of NF-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF or CSF-1), as well as integrin and immunoreceptor-like adhesion signals and interactions, which are provided by osteoclastogenesis-supporting cells, such as osteoblasts, osteocytes and other stromal cells under physiological conditions (Boyle et al., 2003). The early phase of osteoclast differentiation is characterized by the expression of osteoclast-specific genes, such as tartrate-resistant acidic phosphatase (TRAP) in committed precursors (preosteoclasts). Fusion of these preosteoclasts will then lead to formation of large, multinucleated osteoclasts. These giant polykarions spread over the bone surface and digest the underlying bone tissue through the simultaneous release of hydrochloric acid and digestive enzymes onto the bone (Teitelbaum, 2000).

The tightly regulated balance between bone resorption and bone formation can be altered under pathological conditions. Enhanced maturation and activation of osteoclasts leads to pathological bone resorption as seen in osteoporosis and inflammatory bone diseases (Győri and Mócsai, 2015). During the pathogenesis of inflammatory bone diseases such as rheumatoid arthritis, gout and periodontitis, the chronic inflammation irreversibly affects the surrounding bone tissue. Bone degradation in inflammatory arthritis is best characterized in human rheumatoid arthritis (Győri and Mócsai, 2015). Rheumatoid arthritis is a chronic autoimmune disease, eventually leading to the destruction of surface cartilage and subchondral bone primarily in the small synovial joints of the hands and feet (Firestein, 2003). Osteoclasts play a key role in the pathogenesis of rheumatoid arthritis (McInnes and Schett, 2007) and numerous studies focused on the crosstalk between osteoclast and the immune system in rheumatoid arthritis. Mature osteoclasts are present at the sites of bone destruction and osteoclastogenesis is enhanced in the close proximity of the inflamed joints (Schett and Teitelbaum, 2009). This pronounced osteoclast formation is due to the accumulation of osteoclast precursors at the sites of erosion and enhanced maturation of these preosteoclasts to bone-resorbing polykarions in the presence of osteoclastogenic cytokines derived from the immune and stromal cells (Schett, 2009). Besides inflammatory bone diseases and osteoporosis, the third major disease where excessive bone loss occurs due to hyperactivation of osteoclasts is tumor-induced osteolysis and the formation of bone metastases.

ROLE OF OSTEOCLASTS IN TUMOR-INDUCED OSTEOLYSIS AND BONE METASTASIS FORMATION

Bone tissue is one of the most common sites for metastasis formation by a large number of solid tumors including lung, prostate, breast, thyroid, colorectal, ovarian cancers, and malignant melanoma. Further, two-third of patients with stage II/III prostate and breast cancers develop bone metastasis (Hernandez et al., 2018). Bone metastases are classified as osteolytic, osteoblastic and mixed lesions. The role of osteoclasts and induction of osteoclastogenesis is best described in the process of osteolytic bone metastasis formation. The presence of osteolytic bone lesions are associated with a set of different morbidities including pathological fractures, pain and hypercalcemia (Weilbaecher et al., 2011), seriously affecting the patient's wellbeing and life expectancy (Coleman and Rubens, 1987).

Although solid tumors capable of forming osteolytic lesions have proteolytic activity, the extent of this is far from being able to break down the bone matrix. Degradation of both the organic and inorganic components of the bone is therefore carried out by osteoclasts, the unique bone-resorbing cells, accumulating in the vicinity of tumor cells forming osteolytic metastases (Croucher et al., 2016). Tumor cells can promote osteoclast-mediated osteolysis via several mechanisms. Either tumor cells can induce osteoclast differentiation directly via the expression of RANKL or they can stimulate osteoclastogenesis indirectly via the activation of osteoblasts (Mundy, 2002). During this latter process, a wide range of tumor cell-derived growth factors such as parathyroid hormone related peptide – (PTHrP) can induce the expression of RANKL on osteoblasts, which in turn drives the differentiation of multinucleated osteoclasts from myeloid precursors (Suva et al., 1987). Mature osteoclasts then resorb the bone matrix and allow tumor cells to grow and spread within the tissue.

Skeletal metastases formation is a self-perpetuating cycle where tumor cells and bone-resorbing osteoclasts are enrolled in a "vicious" cycle characterized by the release of bone-stored growth factors by osteoclast-mediated bone resorption, which further stimulates cancer cell survival and proliferation (Faccio, 2011). Malignant cells express a wide range of growth factors and cytokines, which can directly or indirectly activate osteoclasts. On the other hand, osteoclast-mediated bone resorption can lead to the release of bone-stored cytokines including TGFβ, which are able to promote cancer cell survival and growth (Kakonen et al., 2002). Bone matrix-derived cytokines can also provide a chemotactic stimulus for directed cancer cell migration (Orr et al., 1979). Further, RANKL itself via a paracrine mechanism can serve as a chemoattractant and increase migration of RANK-positive cancer cells (Jones et al., 2006). As a consequence, osteoclast-mediated osteolysis results in an altered bone microenvironment, which facilitates cancer growth and metastasis formation. Later during the disease, these interactions between tumor and bone cells result in a locked cycle of tissue destruction and cancer growth ("vicious cycle" of bone metastasis formation) (Roodman and Dougall, 2008). In line with this, it has been found in mice, that cancer cells, which are more closely located to the bone surface showed increased proliferation compared to the ones distant from the bone (Kostenuik et al., 1992). Underlying the role of osteoclasts in the process of bone metastasis formation, bisphosphonate pyrophosphate analogs that target osteoclasts are used to prevent bone destruction

and modify progression of skeletal metastasis in cancer (Choi et al., 2009).

Bone remodeling is also closely coupled with the lympho-hematopoietic system. The similarity between the signaling mechanism of the bone and immune systems in this shared microenvironment indicate that cancer cell growth associated with osteolytic bone degradation can also drive local immunosuppression and accumulation of metastasis-promoting immune cell populations (Lorenzo et al., 2007). A large body of experimental evidence has implicated the role of the immune system in the regulation of bone homeostasis both in humans and mice (Lorenzo et al., 2010).

INTERPLAY BETWEEN THE SKELETAL AND IMMUNE SYSTEMS DURING BONE METASTASIS FORMATION

Tumor development can alter both the skeletal and immune homeostasis (Nakashima and Takayanagi, 2009). Malignant cells are able to suppresses certain effector immune cells subsets, such as conventional $CD8^+$ T cells, which can recognize and kill cancer cells (Schreiber et al., 2011). Other immune cells, such as regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSC) and tumor-associated macrophages (TAMs) also play important roles in promoting cancer growth and metastasis formation. On the other hand, the cellular elements and humoral factors of the innate and adaptive immune systems can affect osteoclastogenesis as well (Takayanagi, 2010). Macrophages and dendritic cells also share common precursors with osteoclasts, which underline the importance of the field of osteoimmunology (Takayanagi, 2007).

While Th1 and Th2 cytokines exert an inhibitory effect on osteoclastogenesis, the IL-17 producing T helper type 17 (Th17) cells have been described to be highly osteoclastogenic (Sato et al., 2006b). Th17 cells can express high levels of RANKL and as a consequence directly promote osteoclastogenesis. Moreover, they may activate inflammation locally, leading to the release of proinflammatory mediators (e.g., TNF-α, IL-1, and IL-6), which can potentiate RANKL expression on osteoclastogenesis-supporting cells (Sato et al., 2006b). Further, Th17 cells were described to activate osteoclastogenesis-driven osteolysis through RANKL production during inflammatory arthritis (Okamoto and Takayanagi, 2011). However, no direct role for Th17 cells in cancer induced bone disease has been reported so far. In line with this, IL-17F, which shows 50% homology with IL-17A and shares its receptor, was produced in high levels in the 4T1 preclinical tumor model, but found not to be necessary for the development of pre-metastatic bone disease (Monteiro et al., 2013).

The effects of conventional T cells on osteoclastogenesis are normally suppressed by regulatory T (Treg) cells. These cells are able to inhibit osteoclast development and function via the release of tumor growth factor-β (TGF-β), IL-10 (Kim et al., 2007b; Kelchtermans et al., 2009) and expression of CTLA-4 (Zaiss et al., 2007). In addition to their suppressive capabilities, tumor-infiltrating Treg cells have also been described to express RANKL (Tan et al., 2011). As a consequence, the effect of Treg cells on osteoclastogenesis depends on the balance between positive and negative factors within the tumor microenvironment.

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells, which are capable of potently suppressing the anti-tumor functions of conventional T lymphocytes (Gabrilovich and Nagaraj, 2009). It has been shown that MDSCs derived from the tumor microenvironment can differentiate into bone-resorbing osteoclasts under tissue culture conditions (Sawant et al., 2013) and MDSCs from tumor-bearing mice have increased osteoclastogenic potential (Zhuang et al., 2012).

Tumor-associated macrophages (TAMs) are a predominant white blood cell subset both in the bone and tumor microenvironment, which can influence tumor development, proliferation, growth, survival, and metastasis formation. Macrophages classically have been divided into proinflammatory M1 and anti-inflammatory M2 subsets (Mosser and Edwards, 2008). When activated, M1 macrophages secrete high levels of proinflammatory cytokines and participate in the elimination of tumor cells (Gabrilovich et al., 2012). However, M2 macrophages are characterized by high expression of mannose receptors, scavenger receptors and IL-1Ra (Zhang et al., 2012), and are also often found in human solid tumors. Activated M2 macrophages generate high levels of IL-10 and TGF-β, which can suppress $CD4^+$ and $CD8^+$ conventional T cells (Biswas and Mantovani, 2010). In preclinical studies, where tumor-associated macrophages were depleted using clodronate liposomes, reduced number of bone metastatic lesions were detected (Hiraoka et al., 2008).

ROLE OF RANKL SIGNALING IN OSTEOCLASTS AND BONE METASTASIS FORMATION

Receptor activator of NF-κB ligand (RANKL) belongs to the tumor necrosis factor (TNF) superfamily of cytokines and it is expressed by monocytes, T and B cells, dendritic cells and osteoclastogenesis-supporting cells, such as osteoblasts and synovial fibroblasts (Caetano-Lopes et al., 2009). Parathyroid hormone, 1,25-dihydroxy-cholecalciferol (active vitamin D3) and prostaglandins can promote the secretion of RANKL by osteoblasts and other stromal cellular elements (Takayanagi, 2007). $CD4^+$ conventional and regulatory T lymphocytes are also able to provide RANKL in membrane-bound form as well as release it in a soluble form (Wong et al., 1997). TNF-α, IL-1, IL-6 and IL-17 cytokines can increase RANKL expression on osteoclastogenesis-supporting cells, thereby stimulating RANKL signaling (Takayanagi, 2007). Osteoprotegerin (OPG), expressed by osteoblasts and other stromal cells, is a soluble decoy receptor for RANKL capable of inhibiting RANK signaling (Simonet et al., 1997).

The RANKL receptor, RANK, is highly expressed by preosteoclast. RANKL binding to RANK leads to receptor trimerization and activation of the adapter protein TRAF6, which further stimulates transcription factor NF-κB and members of the mitogen-activated protein kinase (MAPK)

family (Takayanagi, 2010) as shown on **Figure 1**. Nuclear factor of activated T-cell cytoplasmic 1 (NFATc1), the master regulator of osteoclast differentiation, is also activated by RANK receptor signaling (Takayanagi, 2010). NFATc1 translocates to the nucleus and then amplifies its own expression resulting in strong induction of NFATc1 expression (Asagiri et al., 2005). Generation of calcium signal and calcineurin activation are also important for NFATc1 induction. NFATc1 together with activator protein 1 (AP-1) and microphthalmia-associated transcription factor (MITF) induce then the expression of osteoclast-specific genes encoding for tartrate-resistant acid phosphatase (TRAP), cathepsin K (CTSK) and the β3 integrin (Takayanagi, 2007).

The expression of NFATc1 in osteoclasts and their precursors is also regulated on the epigenetic level. An important step in the differentiation of osteoclasts occurs at the NFATc1 promoter when the histone methylation changes from H3K4me3/H3K27me3 to H3K4me3 (Yasui et al., 2011). A histone demethylase, Jmjd3 converts the bivalent H3K4/H3K27 trimethylation to monovalent H3K4me3 in preosteoclasts following RANKL stimulation, leading to increased osteoclast differentiation as well (Yasui et al., 2011). On the other hand, NFATc1 expression can also be inhibited by other transcription factors such as Interferon regulatory factor 8 (IRF-8) (Zhao et al., 2009), bZIP motif containing transcription factor MafB (Kim et al., 2007a), B-cell lymphoma 6 (Bcl-6) (Miyauchi et al., 2010), and Leukemia/lymphoma related factor (LRF) (Tsuji-Takechi et al., 2012). The expression of those transcription factors decrease during osteoclast differentiation, which is mediated by the DNA methyl-transferase 3A (Dnmt3A) (Nishikawa et al., 2015). Furthermore, the B lymphocyte-induced maturation protein (Blimp) was shown to be able to inhibit IRF-8, MafB, Bcl-6 and LRZ transcription factors, leading to upregulated NFATc1 expression and enhanced osteoclastogenesis (Nishikawa et al., 2010). In line with this, Blimp1-deficient mice exhibit increased bone mass and osteopetrotic disease (Nishikawa et al., 2010).

Cancer cells enhance osteoclast-driven osteolysis via several different mechanisms. Upregulation of the expression of RANKL on osteoclastogenesis-supporting cells, downregulation of OPG expression or increased secretion of factors activating RANK receptor signaling have all been described in the context of breast cancer bone metastases (Kearns et al., 2008). Prostate tumor cells can even express RANKL themselves (Brown et al., 2001). Further, secretion of RANKL has also been described by multiple myeloma cells (Farrugia et al., 2003; Sezer et al., 2003). While breast tumors do not upregulate RANKL (Thomas et al., 1999), those cells can eventually induce the expression of RANKL on osteoblasts (Kitazawa and Kitazawa, 2002) and other osteoclastogenesis-supporting cells via the production and release of PTHrP (Mancino et al., 2001). RANK receptor expression by melanoma, breast and prostate cancer cell lines has also been described (Jones et al., 2006), and involved in the autocrine effect of tumor cell-derived RANKL on promoting cancer cell migration. Downregulation of OPG secretion has been found to be characteristic for breast cancer and multiple myeloma cells (Thomas et al., 1999; Giuliani et al., 2001). As a consequence, the RANKL-OPG balance is disturbed within the bone microenvironment in favor of supporting osteoclast-induced osteolysis and bone metastasis formation (Grimaud et al., 2003). Denosumab, a monoclonal antibody raised against RANKL, demonstrated efficacy in preventing tumor-induced bone loss in patients with skeletal metastasis (Choi et al., 2009).

It has also been reported that certain tumor cell-derived soluble factors are able to induce osteoclastogenesis independent of RANKL. Secretion of lysyl oxidase (LOX) from primary breast carcinoma cells induced osteoclast differentiation and osteolytic skeletal lesion formation in animal tumor models (Cox et al., 2015). However, LOX failed to substitute for RANKL when Tnfrsf11a (RANK)-deficient bone marrow cells were treated with recombinant LOX protein in subsequent experiments (Tsukasaki et al., 2017). Similarly, initial data indicated that Tnfrsf11a$^{-/-}$ primary bone marrow cells were capable to differentiate into osteoclasts under TNFα stimulation, when RBP-J transcription factor was deleted in the progenitors (Kim et al., 2005; Zhao et al., 2012). However, RBP-J deletion in mice did not exhibit obvious defects in bone phenotype, suggesting that RBP-J plays a minor role in osteoclast development under physiological conditions (Zhao et al., 2012). Although there have been other factors indicated to induce RANKL-independent osteoclastogenesis, they may be able to promote osteoclast differentiation only under certain conditions and cannot completely substitute for RANK ligand (Tanaka, 2017). Identifying the precise role of these factors in osteoclasts within the solid tumor microenvironment requires further investigation.

ROLE OF CSF-1 SIGNALING IN OSTEOCLASTS AND BONE METASTASIS FORMATION

Another key osteoclastogenic cytokine that governs osteoclastogenesis is CSF-1 (or macrophage-colony stimulating factor, M-CSF). CSF-1 is a polypeptide growth factor that binds to its plasmamembrane receptor (CSF-1R) encoded by the c-fms gene (Stanley et al., 1983). CSF-1 is essential for the development and survival of preosteoclasts (Ross and Teitelbaum, 2005). CSF-1 was originally identified as a regulator of macrophages and their bone marrow precursors (Stanley et al., 1983). As shown on **Figure 1**, once CSF-1 binds to its transmembrane receptor CSF-1R, it induces tyrosine phosphorylation of the cytoplasmic domain of the receptor (Pixley and Stanley, 2004). Then Src homology 2 domain (SH2)-bearing adapter molecules (such as Grb2) are recruited to the phosphorylated tyrosine residues and initiate different signaling cascades (including the MAP-kinase cascade) that lead to cell proliferation and differentiation (Pixley and Stanley, 2004). CSF-1 is also known to induce cytoskeletal rearrangement in osteoclasts by activation of the c-Src and phosphoinositide 3-kinases (Nakamura et al., 1995; Insogna et al., 1997; Pilkington et al., 1998). CSF-1 deficient mice are osteopetrotic (Wiktor-Jedrzejczak et al., 1990).

When considering CSF-1R and RANK-driven non-pathological osteoclastogenesis, it is important to note, that apart from TGF-β, the cytokine IL-1 is supposed to be released from the bone as an autocrine factor. It has been described,

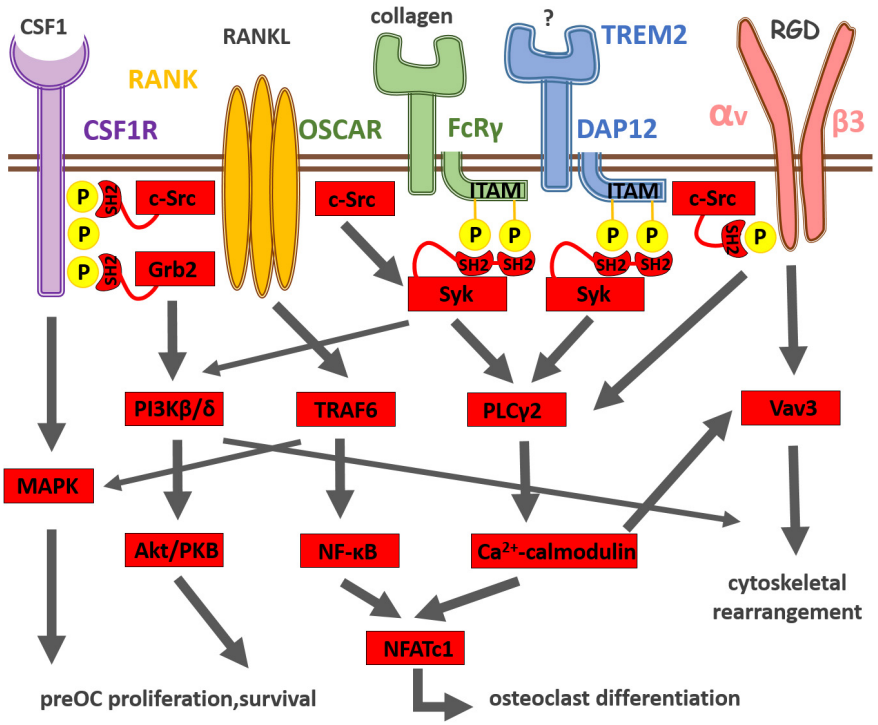

FIGURE 1 | Summary of osteoclast differentiation induced by integration of CSF-1R, RANK, immunoreceptor-like and integrin receptor signaling. CSF-1 and its receptor CSF-1R activate the MAPK cascade pathway leading to the survival and proliferation of preosteoclasts. RANKL and its receptor RANK transduce signals via the adaptor molecule TRAF6, which activates the NF-κB and MAPK pathways leading to the differentiation of osteoclasts. The expression of the master regulator of osteoclastogenesis, NFATc1, is driven by NF-κB and NFATc1. The activation of NFATc1 is also regulated by the costimulatory signaling pathways, where FcRγ, DAP12 and their associating partners (OSCAR and TREM2, respectively) recruit Syk, which further activates PLCγ2, resulting in the activation of calcium signaling. The calcium signaling activates then calcineurin, which in turn promotes NFATc1 expression. The calcium signal also induces Vav3 activation involved in αvβ3 integrin signaling, which leads to cytoskeletal reorganization and osteoclastic bone resorption.

that osteoclast precursor interaction with bone matrix (but not plastic surface) can induce osteoclast formation directly by an interleukin-1-mediated autocrine mechanism in the presence of CSF-1 (Yao et al., 2008; De Vries et al., 2015).

In addition to its physiological role in myeloid cells, increased expression of CSF-1 has been detected in breast, colorectal, ovarian and uterine cancers, where the extent of its expression correlates with poor prognosis (Kacinski, 1995; Smith et al., 1995). Furthermore, in human breast carcinomas, overexpression of CSF-1 and its receptor positively correlates with high grade invasiveness (Kacinski, 1995; Smith et al., 1995). Importantly, a strong correlation of CSF-1 expression with CSF-1R-positive tumor-associated macrophage infiltration has also been detected in human carcinomas (Tang et al., 1990; Scholl et al., 1994). We recently demonstrated that pharmacological inhibition or genetic ablation of CSF1 in cancer cells reduces the accumulation of immunosuppressive CSF-1R$^+$ tumor-associated macrophages and increases CD8$^+$ T cell attack on tumors (Győri et al., 2018).

In line with this, it is highly likely that certain tumor cells capable of forming osteolytic bone metastasis recruit not only tumor-associated macrophages, but monocytes/osteoclast precursors via the secretion of CSF-1 into the tumor microenvironment. Furthermore, tumor cell-derived CSF-1 can also enhance the maturation of those precursors to bone-resorbing osteoclasts in the presence of an osteoclastogenic milieu. As a consequence, pharmacological blockade of CSF-1 might offer benefits for patients with osteolytic bone metastases and CSF-1 inhibitors are being evaluated in clinical trials. PLX3397, an orally available CSF-1R inhibitor is currently being administered in clinical trials and early data suggested good tolerability and beneficial effects (Ries et al., 2015). It has also been described, that CSF-1R and αvβ3 integrins collaborate during osteoclast differentiation via shared activation of downstream signaling pathways (Faccio et al., 2003; Ross and Teitelbaum, 2005).

ROLE OF INTEGRIN SIGNALING IN OSTEOCLASTS AND BONE METASTASIS FORMATION

Integrins are heterodimeric transmembrane proteins that facilitate cell-cell and cell-matrix interactions (Schwartz et al., 1995). Integrins can activate many intracellular signaling pathways and induce the proliferation, survival, and cytoskeletal rearrangements of the target cells (Hynes, 1992). So far 8 beta and 18 alpha integrin subunits have been described, which can

combine into 24 unique heterodimers within the different cell types, each of those characterized by different ligand binding characteristics, signaling and regulatory mechanisms (Hynes, 2002). Integrin heterodimers can be activated by conformational changes in their extracellular domains. When inactive, integrin dimers are found in a closed conformation within the cell membrane. Upon activation through the cytoplasmic domains, the α and β cytoplasmic and transmembrane regions become separated resulting in the unfolding of the extracellular ligand binding domain (inside-out signaling) (Shattil et al., 2010). This open conformation then promotes extracellular ligand binding and initiates integrin-driven intracellular signaling pathways (outside-in signaling) (Qin et al., 2004).

Integrins play critical roles on both tumor cells and osteoclasts in promoting bone metastasis formation. During the process of bone resorption, mature osteoclasts attach to the bone surface, generate an actin ring mediated sealing zone and then secrete hydrochloric acid and enzymes to lyse the underlying bone matrix. All of these steps at least in part are regulated by integrin heterodimers located on the surface of osteoclasts (Teitelbaum and Ross, 2003). Different integrins are involved in the binding of osteoclasts to the bone, including αvβ3, αvβ5, and α2β1, of which, αvβ3 is the dominant integrin on osteoclasts (Ross and Teitelbaum, 2005; Novack and Teitelbaum, 2008; Ross et al., 1993). αvβ3 integrins are responsible for mediating osteoclast-bone matrix recognition and subsequent attachment to the bone surface (Ross et al., 1993). αvβ3 integrin signaling is essential for osteoclast spreading and to create the characteristic ruffled border of the plasmamembrane for subsequent resorption (Faccio et al., 2003; McHugh, 2000). Furthermore, osteopontin binding to αvβ3 induce podosome formation and cytoskeletal rearrangement (Miyauchi et al., 1991). Additional studies identified further downstream signaling effector molecules such as Vav, a critical regulator of osteoclast differentiation and actin ring formation (Faccio et al., 2005). As a consequence, mice with genetic inactivation of β3 integrin ($\beta3^{-/-}$) have defective osteoclast function (McHugh, 2000) and are protected from cancer cell-induced bone loss (Bakewell et al., 2003). αvβ3 integrins are also critical for the activation of c-Src which is a key signaling molecule in osteoclast spreading and cytoskeletal reorganization (Zhang et al., 2000; Faccio et al., 2003).

Cancer cells display altered integrin expression and signaling, allowing them to colonize new tissues by escaping from cell–cell and cell–matrix connections. Maintaining adhesion to the extracellular matrix via integrins is key to cell survival (Frisch and Screaton, 2001). Disruption of cell–cell or cell–matrix interactions lead to loss of survival signals and non-transformed cells which are anchorage-dependent undergo a form of programmed cell death called anoikis (Frisch and Screaton, 2001). Under physiological conditions, this form of apoptosis assures that isolated cells are not able to migrate to inappropriate locations (Frisch and Screaton, 2001). Metastatic cancer cells that can resist anoikis utilize several different mechanisms in order to be able to settle in a novel microenvironment. These mechanisms include altered integrin expression (Bissell and Radisky, 2001), activation of integrin signaling cascade downstream molecules such as focal adhesion (Frisch et al., 1996) and c-Src kinases (Shain et al., 2002), EGFR (Demers et al., 2009), as well as suppression of apoptotic pathways (Simpson et al., 2008). Expression of αvβ3 integrin is elevated on human breast cancer bone metastases (Zhao et al., 2007), and ectopic overexpression of the β3 subunit on breast cancer cells has been demonstrated to enhance tumor establishment in bone (Sloan et al., 2006).

ROLE OF c-Src KINASE IN OSTEOCLASTS AND BONE METASTASIS FORMATION

The non-receptor tyrosine kinase, c-Src is a key signaling molecule in bone metabolism and plays an important role in the regulation of growth, survival, proliferation, adhesion and motility (Brunton and Frame, 2008). Preclinical studies demonstrated an important role for Src-family kinases in osteoclast-mediated bone resorption (Horne et al., 1992). Mature osteoclasts express high levels of c-Src (Miyazaki et al., 2004). Pharmacological inhibition of c-Src kinase-activity decreased preosteoclast migration and inhibited the subsequent formation of resorption pits in *in vitro* studies (de Vries et al., 2009). Mice with genetic inactivation of the c-Src gene exhibit osteopetrosis, a severe disease that makes bones abnormally dense and prone to fractures (Soriano et al., 1991). Since c-Src-deficient mice had normal osteoclast numbers, the osteopetrotic phenotype is rather due to a failure in osteoclast function (Soriano et al., 1991). Further, Src-deficient mature osteoclasts fail to form actin rings and sealing zones (Boyce et al., 1992).

Besides regulating cytoskeletal rearrangement and ruffled border formation, Src-family kinases are also present at vesicular membranes, where they are required for the secretion of hydrochloric acid and bone-degrading enzymes (Furuyama and Fujisawa, 2000; Edwards et al., 2006). Among Src-family kinase binding partners, Tks5 has been shown to mediate podosome formation and cell-cell fusion in osteoclasts (Oikawa et al., 2012). Tks5, which has been originally described as a regulator of invadopodia formation in tumor cells, was reported to be phosphorylated on tyrosine residues in a c-Src-dependent manner within osteoclasts (Oikawa et al., 2012). Furthermore, it was also shown that co-culturing malignant melanoma cells with osteoclasts promoted the formation of melanoma-osteoclast hybrid cells (Oikawa et al., 2012). Fusion of osteoclasts with cancer cells can contribute to increased bone resorption activity, secretion of chemokines promoting osteolytic bone metastasis formation and even the evasion of immune surveillance (Oikawa et al., 2012).

An elevated level of activity of c-Src is suggested to be linked to cancer progression and a large body of evidence suggests that Src-family kinase has a critical role in cancer growth and invasion (Thomas and Brugge, 1997). Further, Src expression positively correlates with the metastatic spread of cancer cells (Boyer and Poupon, 2002). Importantly, correlation was also observed between tumor cell colonization in bone and Src kinase activity (Myoui et al., 2003). Increased Src family kinase activity fueled tumor cell growth and enhanced parathyroid hormone related peptide (PTHrP) release

within bone metastases (Myoui et al., 2003). Small-molecule kinase inhibitors are emerging new therapeutics in diseases with pathological bone loss and have potential for the treatment of bone metastases as well. Preclinical studies with therapeutic Src inhibitors (dasatinib, saracatinib, and bosutinib) demonstrated anti-tumor and anti-osteoclast effects, as well as clinical studies provided evidence that Src-family kinase inhibitors might be beneficial for patients with refractory disease (Boyce and Xing, 2011).

ROLE OF IMMUNORECEPTOR-LIKE SIGNALING IN OSTEOCLASTS AND BONE METASTASIS FORMATION

Classical immunoreceptors, such as T and B cell receptors (TCR, BCR, respectively) as well as Fc-receptors (FcR) use a common signaling machinery within the innate and adaptive immune systems. Firstly, when ligand binds to the classical immunoreceptor, tyrosine residues within the immunoreceptor tyrosine-based activation motifs (ITAMs) are phosphorylated by Src kinases as shown on **Figure 1**. This then results in the SH2-domain dependent recruitment of the spleen tyrosine kinase (Syk) (Fodor et al., 2006). Src and Syk non-receptor tyrosine kinases then activate downstream effector molecules such as the phospholipase Cγ2 (PLCγ2) and phosphoinositide 3-kinase (PI3K) isoforms. We and others recently recognized that this classical immunoreceptor-like signaling mechanism is present in a range of non-lymphoid cell types (e.g., osteoclasts) too (Koga et al., 2004; Mócsai et al., 2004).

Osteoclasts carry at least two ITAM sequence-containing adapter molecules, namely the DAP12 and the FcR γ-chain (FcRγ). These proteins likely work together with adhesion receptors OSCAR and TREM2 on osteoclasts (Koga et al., 2004). Deletion of DAP12 and TREM2 in mice results in failure of osteoclast differentiation and function (Paloneva et al., 2003; Humphrey et al., 2004). On the other hand, TREM2 and DAP12 deficient mice are not osteopetrotic, which indicates that osteoclastogenesis can proceed through a mechanism that requires the other ITAM-bearing molecule FcR γ-chain. FcRγ, may be able to compensate for the lack of DAP12, since double mutant mice for FcRγ and DAP12 are severely osteopetrotic (Koga et al., 2004; Mócsai et al., 2004). These ITAM-bearing co-receptors most likely mediate osteoclast-osteoblast and osteoclast-bone matrix interactions together with integrin adhesion receptors (Koga et al., 2004; Mócsai et al., 2004). More recently, paired immunoglobulin-like receptor-A (PIR-A) and osteoclast-associated receptor (OSCAR) has been shown to associate with the FcRγ-chain (FcRγ), while DAP12 interaction with signal regulatory protein β1 (SIRPβ1), sialic acid-binding immunoglobulin-like lectin 15 (Siglec-15) and myeloid DAP12-associating lectin (MDL)-1 besides TREM2 has also been reported (Koga et al., 2004; Mócsai et al., 2004; Joyce-Shaikh et al., 2010; Kameda et al., 2013; Negishi-Koga et al., 2015).

As discussed before, β3 integrin ligation leads to activation of Src-family kinases and a non-receptor kinase, Syk. The Syk tyrosine kinase is required for the differentiation and function of osteoclasts as well (Mócsai et al., 2004). Activation of Syk requires the presence of ITAM-bearing adapters, DAP12 and FcRγ-chain in the osteoclast (Mócsai et al., 2004). We recently showed, that osteoclast-specific genetic inactivation of Syk also leads to elevated bone volume in mice (Csete et al., 2019). Syk inhibitors (like fostamatinib) has shown promising effects in rheumatoid arthritis and may also provide significant bone protection in a tumor-associated environment (Mócsai et al., 2010). The osteopetrotic phenotype (Soriano et al., 1991) and defective osteoclastogenesis in the Src-deficient mice indicates that Src-family kinases might be also required for the phosphorylation of DAP12 and FcRγ in osteoclasts (Lowe et al., 1993). Syk then promotes the formation of the Bruton's tyrosine kinase (Btk)/B cell linker protein (BLNK)/SH2 domain-containing leukocyte protein of 76 kDa (SLP76) complex, which activates the phospholipase Cγ2 (PLCγ2) enzyme. PLCγ2 cleaves the plasmamembrane phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (Feng et al., 2012). We and others showed that PLCγ2 is needed for the differentiation and function of osteoclasts and PLCγ2$^{-/-}$ mice have increased bone mass (Mao et al., 2006; Chen et al., 2008; Epple et al., 2008; Kertész et al., 2012). IP_3 generated by PLCγ2 then binds to its receptor, which in turn induces the release of calcium ions from the sarco/endoplasmic reticulum (SR/ER). Sarco/endoplasmic reticulum calcium ATPase 2 (SERCA2) re-uptakes the calcium into SR/ER (Yang et al., 2009). Repetitive influx and efflux of calcium ions results in calcium oscillations within the cytoplasm of the osteoclast. Calcium oscillations activate calcineurin, which leads to the dephosphorylation of the master regulator of osteoclastogenesis, NFATc1, enabling its autoamplification and entry to the nucleus. Calcium signaling can also induce c-Fos transcription factor via Calcium/calmodulin dependent kinase (CAMK) IV and cAMP response-element binding protein (CREB) pathways (Sato et al., 2006a). Further, transmembrane protein 64 (Tmem64) has been reported to be essential for the function of SERCA2 (Kim et al., 2013), while the transmembrane protein 178 (Tmem178) was shown to be required to suppress the excessive efflux of calcium in a PLCγ2-dependent manner (Decker et al., 2015). Accordingly, mice deficient in DAP12/FcRγ, SERCA2 and Tmem64 are osteopetrotic and exhibit defective calcium oscillations (Yang et al., 2009; Kim et al., 2013). On the other hand, lack of Tmem178 in mice results in an osteopenic phenotype with increased amplitude of calcium oscillations (Decker et al., 2015).

Immunoreceptor-like signaling has been implicated in metastatic spread and homing of cancer cells to distant organs such as the bone. TREM2 was found to be upregulated on peripheral blood monocytes in tumor-bearing hosts and elevated expression levels in pulmonary macrophages correlated with high pathological stage of lung adenocarcinomas (Yao et al., 2016). Further, DAP12 expression in colorectal cancer cells was associated with higher tumor grade (Shabo et al., 2013) and Syk is also frequently upregulated in recurrent ovarian carcinomas metastasizing to the bone (Yu et al., 2019). On the other hand, while PLCγ2 has been implicated in the proliferation and migration of several human cancers (Feng et al., 2012), its genetic

deletion in mice resulted in increased tumor growth (Zhang et al., 2011). Interestingly, it has been shown that PLCγ2$^{-/-}$ mice exhibit increased tumor mass in the bone, despite the decreased osteoclast numbers. Although PLCγ2 is not needed for T cell receptor signaling, altered CD8^{+} T cell activation was observed in PLCγ2$^{-/-}$ tumor-bearing mice (Zhang et al., 2011). Recent evidence indicated that downregulation of PLCγ2 signaling results in the accumulation of immunosuppressive MDSCs in tumor-bearing mice instead (Capietto et al., 2013). This study highlights the role of a permissive immune phenotype and microenvironment as the requirement for osteoclast-mediated promotion of tumor growth and bone metastasis.

ROLE OF EPHRINS AND SEMAPHORINS IN OSTEOCLASTS AND BONE METASTASIS FORMATION

Cell–cell interactions are critical for normal bone homeostasis. Ephrins and Semaphorins are known to be expressed in several cell types and regulate intercellular communication. Eph tyrosine kinase receptors and Ephrin ligands mediate interactions between bone cells through a bidirectional signaling. These membrane-bound proteins have been demonstrated to play important roles in osteoblasts and osteoclasts (Edwards and Mundy, 2008). EphrinB2 has been shown to be regulated by PTH and PTHrP in osteoblasts, while the role of EphrinA4 in modulating osteoclast activity via β3-integrin signaling has recently been identified (Stiffel et al., 2014). The altered expression of Eph tyrosine kinase receptors and ephrins has also been demonstrated in several human cancers, e.g., EphrinA2 levels were detected to be elevated in late-stage and bone metastatic prostate cancers (Lin et al., 2012).

Semaphorins play critical roles in the immune system, organ and tumor development. It has been shown that Semaphorin 3A (Sema3A) and its receptor neuropilin-1 (Nrp1) suppress osteoclast differentiation and protect mice from excessive bone loss (Hayashi et al., 2012). The Semaphorin 6D (Sema6D), which associate with TREM2/DAP12 via its receptor plexin-A1 (PlxnA1), on the other hand, promoted osteoclast differentiation and function (Takegahara et al., 2006). Semaphorins were also shown to contribute to the pathogenesis of cancer, including the regulation of epithelial-mesenchymal transitions and stem cell properties (Lontos et al., 2018). In the process of bone metastasis formation Semaphorin 4D, a coupling factor expressed on osteoclasts that inhibits osteoblast differentiation, has recently been implicated (Lontos et al., 2018).

ROLE OF PHOSPHOINOSITIDE 3-KINASES IN OSTEOCLASTS AND BONE METASTASIS FORMATION

Phosphoinositide 3-kinases (PI3Ks) generate 3-phosphoinositide lipids in cell membranes and play important roles in key biological functions including cellular proliferation, survival, cytoskeletal reorganization, migration, metabolism and vesicular trafficking (Vanhaesebroeck et al., 2012; Hawkins and Stephens, 2015). PI3Ks are subgrouped into three unique classes of which the Class I. PI3K family comprise of PI3Kα, PI3Kβ, PI3Kγ, and PI3Kδ (Hawkins et al., 2006; Vanhaesebroeck et al., 2010). PI3Kα and PI3Kβ are ubiquitously expressed, while the PI3Kγ and PI3Kδ isoforms are mainly restricted to hematopoietic lineages, e.g., white blood cells (Okkenhaug, 2013). 3-phosphoinositide lipids such as PIP_3 can initiate a number of downstream signaling pathways and activate effector molecules, like the 3-phosphoinositide dependent protein kinase-1 (PDK1), Ser/Thr kinase Akt (or protein kinase B) and the mammalian target of rapamycin complex 1 (mTORC1) (Hawkins et al., 2006).

PI3-kinases are central downstream effectors of the RANK, CSF-1 and αvβ3 integrin receptors in osteoclasts. Activation of Class I. PI3Ks downstream of those receptors results in the generation of PIP_3 from PIP_2. Class I.A PI3Ks (PI3Kα, PI3Kβ, PI3Kδ) are designated based on catalytic subunits p110α, p110β, and p110δ, which associate with regulatory subunits p85α, p85β, and p55γ. The sole Class I.B member PI3Kγ consists of catalytic subunit p110γ and regulatory subunit p101 or p84. Mice lacking p110α exhibit embryonic lethality (Bi et al., 1999), while p110γ mutant mice are viable but show defects in immune cell proliferation and function (Sasaki et al., 2000). Mice knockout for the p85α/β subunit showed impaired osteoclast differentiation and ruffled border formation (Munugalavadla et al., 2008; Shinohara et al., 2012). These data indicated that PIP_3 is a strong inducer of osteoclast differentiation and PI3-kinases might play an important role in regulating osteoclastogenesis.

We and others have shown that Class I. PI3Ks play a critical role in osteoclasts and could be potential therapeutic targets in bone metastases (Shinohara et al., 2012; Shugg et al., 2013; Győri et al., 2014). Previous studies using non-selective PI3K inhibitors (such as wortmannin) indicated a role for PI3-kinases in osteoclastogenesis (Hall et al., 1995; Nakamura et al., 1995; Sato et al., 1996). Using pharmacological and genetic approaches we demonstrated that PI3Kβ is important for normal osteoclast differentiation and function (Győri et al., 2014). PI3Kβ$^{-/-}$ mice had increased bone mass and PI3Kβ$^{-/-}$ osteoclasts displayed altered morphology *in vivo* (Győri et al., 2014). Further, PI3Kβ$^{-/-}$ osteoclasts were unable to form actin rings and to release intracellular vesicles and cathepsin K (Győri et al., 2014). In addition to this, the PI3Kδ isoform, which is mainly expressed in hematopoietic cells, has been found to be an attractive target for anti-resorptive agents since recent data indicated that PI3Kδ regulates osteoclast cytoskeleton and resorptive activity (Shugg et al., 2013).

Class I. PI3Ks are often mutated in human cancers (Fruman and Rommel, 2014). Aberrant activation and amplification of PI3Kα is one of the most frequently observed mutations associated with malignant transformation (Samuels et al., 2005; Thorpe et al., 2015). Oncogenic mutations have also been detected in PI3Kβ, but rarely in PI3Kγ and PI3Kδ (Hill et al., 2010; Dbouk et al., 2013). An elevated level of activity of PI3Kδ was found in non-hematopoietic cell types, including those of breast and melanocytic origin (Sawyer et al., 2003). Further, PI3K downstream targets are also often seen abnormally activated in human malignancies. Overexpression of Akt/PKB has been

observed in a wide range of solid tumors, including breast, ovarian and prostate carcinomas (Scheid and Woodgett, 2001; Vivanco and Sawyers, 2002). The recognition of the tumor suppressor PTEN, a phosphatase for 3-phosphoinositide lipids, proved to be an indicator of aberrant PI3K signal transduction in cancer (Cantley and Neel, 1999). Deletions and mutations in the PTEN gene also lead to the accumulation of PI3K lipid products, which is observed in many human tumors (Di Cristofano and Pandolfi, 2000).

There is substantial interest in the pharmaceutical industry to develop PI3K inhibitors for the treatment of human cancers. To avoid the serious side effects of pan–Class (I) PI3K drugs affecting glucose homeostasis (Busaidy et al., 2012), administration of isoform-selective PI3K inhibitors below their maximum tolerated dose, most likely in combination may be beneficial. Based on its role in the tumor microenvironment PI3Kβ and/or PI3Kδ may be suitable therapeutic targets for bone metastases with tumor-induced osteolysis.

ROLE OF MICRORNAs AND EXTRACELLULAR VESICLES IN OSTEOCLASTS AND BONE METASTASIS FORMATION

MicroRNAs are non-coding RNAs of approximately 20–22 nucleotides in length which regulate critical cellular processes including proliferation, differentiation, and survival. Recent data indicated that microRNAs are involved in tumorigenesis and osteolytic bone metastasis formation as well. Various tumor-cell derived mature microRNAs were implicated in regulating osteoclast differentiation. miR-223 was shown to regulate the level of the CSF1 receptor in preosteoclasts (Sugatani and Hruska, 2007), while miR-503 targets RANK (Chen et al., 2014). Further, miR-29 promotes murine osteoclastogenesis by regulating osteoclast commitment and migration (Franceschetti et al., 2013). On the other hand, miR-124 was detected to suppress transcription factor NFATc1 during osteoclastogenesis (Lee et al., 2013), while miR-155 represses MITF and PU.1 transcription factors (Mann et al., 2010). It has been reported that administration of miR-141 and miR-219 results in a significant drop in the number of bone-resorbing osteoclasts *in vivo* and tumor burden in breast carcinoma-bearing mice (Ell et al., 2013). Similarly, miR-33a suppressed bone metastasis formation by targeting PTHrP and altering the RANKL/OPG ratios in osteoblasts (Kuo et al., 2013).

MicroRNAs were recently revealed to be present in extracellular vesicles (Valadi et al., 2007). Extracellular vesicles are lipid bilayer coated vesicles ranging from approximately 50 to 5000 nm in diameter. Three main types of extracellular vesicles exist: microvesicles, exosomes and apoptotic bodies (Marton et al., 2017). MicroRNA-containing extracellular vesicle secretion depends on various different factors. Numerous studies suggested the involvement of extracellular vesicles secreted by tumor cells in the development of osteolytic bone metastases (Kagiya, 2015). Exosomal miR-21, miR-210 and miR-378 were found to be important for regulating osteoclast differentiation (Kagiya, 2015). Further, miR-16 and miR-378 were detected to be

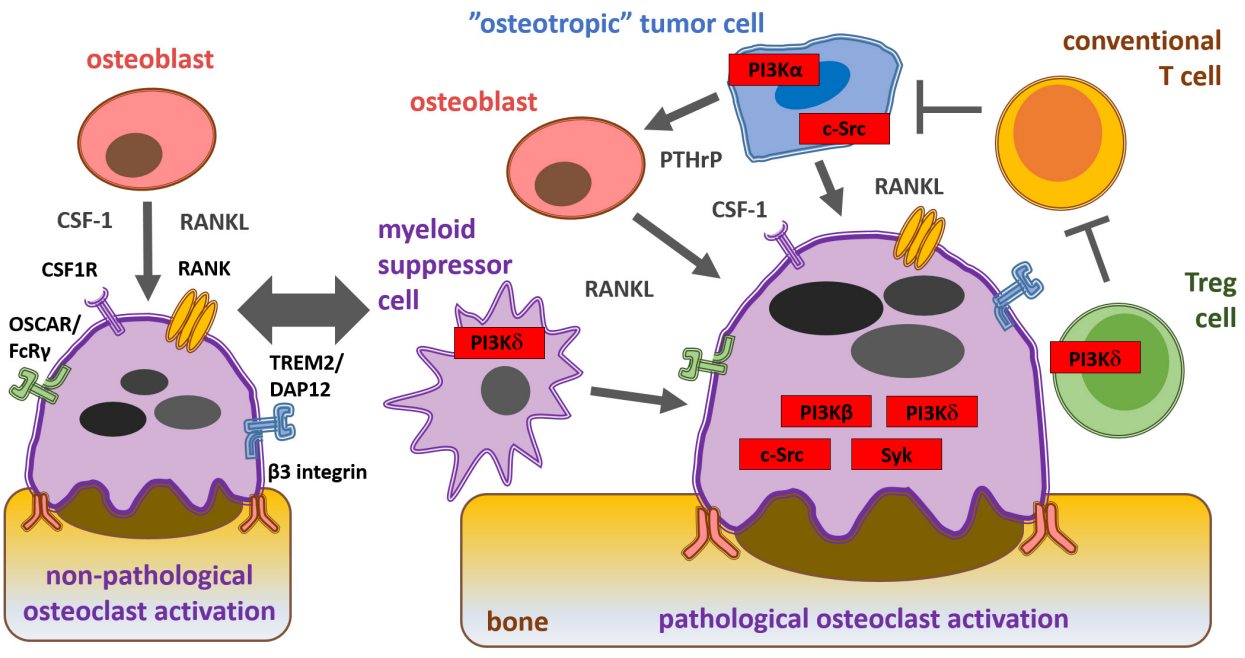

FIGURE 2 | Schematic representation of physiological and pathological osteoclast activation during bone metastasis formation and key signaling molecules within the solid tumor microenvironment from a therapeutic aspect. Osteoblast-derived cytokines and interactions with the bone matrix lead to physiological osteoclast activation via CSF1, RANK, costimulatory and β3 integrin receptors. "Osteotropic" tumor cell recruited regulatory T cells (Tregs) and myeloid derived suppressor cells (MDSC) inhibit conventional T cells (Tconv) and promote cancer cell-induced pathological osteoclast activation via PI3Kβ, PI3Kδ, c-Src, and Syk.

higher in the serum of metastatic breast cancer patients (Arroyo et al., 2011; Ell et al., 2013). Accordingly, extracellular vesicles play an important role in intercellular communication via the transfer of not only microRNAs and proteins, but also bioactive lipids such as PIP_3 generated by phosphoinositide 3-kinases.

THERAPEUTIC ASPECTS AND FUTURE PERSPECTIVES

Tight coupling between bone formation and resorption is essential for bone remodeling. Disruption of this balance can lead to skeletal disorders. Skeletal metastatic disease is a severe consequence of tumor cell dissemination from primary cancer sites, and significantly decreases wellbeing and life expectancy in patients. The bone is a major target for tumor metastasis as it provides a unique environment, which promotes solid cancer cell dissemination (Weilbaecher et al., 2011). The balance between bone-forming osteoblasts and bone-resorbing osteoclasts is altered by bone metastasis formation (Mundy, 2002). Cancer cell invasion into the bone is associated with the activation of osteoclasts. Osteoclast-mediated osteolysis then leads to the release of cytokines and growth factors deposited in the bone matrix. This further fuels tumor growth and dissemination within the tissue ("vicious cycle" of bone metastasis formation) (Faccio, 2011).

RANK ligand, an important osteoclastogenic factor, is expressed by osteoblasts and cancer cells (Kearns et al., 2008). PTHrP secreted by tumor cells indirectly promotes osteoclastogenesis-supporting cells to express RANK ligand (Suva et al., 1987; Mancino et al., 2001). CSF-1, necessary for the development of preosteoclast to osteoclasts, is also derived from cancer cells (Stanley et al., 1983; Smith et al., 1995). Once bone metastasis is established, cancer cells recruit other tumor-associated cells, including fibroblasts and immune cells which secrete cytokines (such as IL-17, IL-1, IL-6, and TNF-α) that increase RANK ligand expression (Mancino et al., 2001; Kitazawa and Kitazawa, 2002). Integrin and immunoreceptor-like signaling leads to the activation of key effector molecules, such as Src-family kinases (Myoui et al., 2003), Syk non-receptor tyrosine kinase (Mócsai et al., 2010) and phosphoinositide 3-kinases (PI3Ks), which further enhance osteoclast activity and subsequent bone destruction (Di Cristofano and Pandolfi, 2000). Summary of the mechanisms of osteoclast activation during cancer cell-induced osteolysis as well as the key osteoclast signaling molecules are shown on **Figure 2**.

Better understanding of the molecular mechanisms governing osteoclast activation in tumor induced-osteolysis and bone metastasis formation may result in the development of novel therapeutic approaches. As several of the osteoclast signal transduction pathways are also activated in cancer cells, they might be tackled synergistically within the solid tumor microenvironment resulting in a parallel reduction in cancer growth and osteoclast-mediated bone loss. On the other hand, a precision medicine-based approach is required to overcome cancer cell resistance mechanisms and to minimize side effects and interference with the mechanisms of physiological bone homeostasis. Taken together, pharmacological targeting of osteoclast and tumor cell signaling pathways may provide novel approaches to improve the long-term survival of cancer patients with bone metastases.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

Arron, J. R., and Choi, Y. (2000). Bone versus immune system. *Nature* 408, 535–536. doi: 10.1038/35046196

Arroyo, J. D., Chevillet, J. R., Kroh, E. M., Ruf, I. K., Pritchard, C. C., Gibson, D. F., et al. (2011). Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc. Natl. Acad. Sci. U.S.A.* 108, 5003–5008. doi: 10.1073/pnas.1019055108

Asagiri, M., Sato, K., Usami, T., Ochi, S., Nishina, H., Yoshida, H., et al. (2005). Autoamplification of NFATc1 expression determines its essential role in bone homeostasis. *J. Exp. Med.* 202, 1261–1269. doi: 10.1084/jem.20051150

Bakewell, S. J., Nestor, P., Prasad, S., Tomasson, M. H., Dowland, N., Mehrotra, M., et al. (2003). Platelet and osteoclast beta3 integrins are critical for bone metastasis. *Proc. Natl. Acad. Sci. U.S.A.* 100, 14205–14210. doi: 10.1073/pnas.2234372100

Bi, L., Okabe, I., Bernard, D. J., Wynshaw-Boris, A., and Nussbaum, R. L. (1999). Proliferative defect and embryonic lethality in mice homozygous for a deletion in the p110alpha subunit of phosphoinositide 3-kinase. *J. Biol. Chem.* 274, 10963–10968. doi: 10.1074/jbc.274.16.10963

Bissell, M. J., and Radisky, D. (2001). Putting tumours in context. *Nat. Rev. Cancer* 1, 46–54. doi: 10.1038/35094059

Biswas, S. K., and Mantovani, A. (2010). Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat. Immunol.* 11, 889–896. doi: 10.1038/ni.1937

Bonewald, L. F. (2011). The amazing osteocyte. *J. Bone Miner. Res.* 26, 229–238. doi: 10.1002/jbmr.320

Boyce, B., and Xing, L. (2011). Src inhibitors in the treatment of metastatic bone disease: rationale and clinical data. *Clin. Investig. (Lond.)* 1, 1695–1706. doi: 10.4155/cli.11.150

Boyce, B. F., Yoneda, T., Lowe, C., Soriano, P., and Mundy, G. R. (1992). Requirement of pp60c-src expression for osteoclasts to form ruffled borders and resorb bone in mice. *J. Clin. Invest.* 90, 1622–1627. doi: 10.1172/jci116032

Boyer, B. Y., and Poupon, M. F. B. (2002). Src kinase contributes to the metastatic spread of carcinoma cells. *Oncogene* 21, 2347–2356. doi: 10.1038/sj.onc.1205298

Boyle, W. J., Simonet, W. S., and Lacey, D. L. (2003). Osteoclast differentiation and activation. *Nature* 423, 337–342. doi: 10.1038/nature01658

Brown, J. M., Corey, E., Lee, Z. D., True, L. D., Yun, T. J., Tondravi, M., et al. (2001). Osteoprotegerin and rank ligand expression in prostate cancer. *Urology* 57, 611–616. doi: 10.1016/s0090-4295(00)01122-5

Brunton, V. G., and Frame, M. C. (2008). Src and focal adhesion kinase as therapeutic targets in cancer. *Curr. Opin. Pharmacol.* 8, 427–432. doi: 10.1016/j.coph.2008.06.012

Busaidy, N., Farooki, A., Dowlati, A., Perentesis, J. P., Dancey, J. E., Doyle, L. A., et al. (2012). Management of metabolic effects associated with anticancer agents targeting the PI3K-Akt-mTOR pathway. *J. Clin. Oncol.* 30, 2919–2928. doi: 10.1200/jco.2011.39.7356

Caetano-Lopes, J., Canhao, H., and Fonseca, J. E. (2009). Osteoimmunology-the hidden immune regulation of bone. *Autoimmun. Rev.* 8, 250–255. doi: 10.1016/j.autrev.2008.07.038

Cantley, L. C., and Neel, B. G. (1999). New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proc. Natl. Acad. Sci. U.S.A.* 96, 4240–4245. doi: 10.1073/pnas.96.8.4240

Capietto, A. H., Kim, S., Sanford, D. E., Linehan, D. C., Hikida, M., Kumosaki, T., et al. (2013). Down-regulation of PLCγ2-β-catenin pathway promotes activation and expansion of myeloid-derived suppressor cells in cancer. *J. Exp. Med.* 210, 2257–2271. doi: 10.1084/jem.20130281

Chen, C., Cheng, P., Xie, H., Zhou, H. D., Wu, X. P., Liao, E. Y., et al. (2014). MiR-503 regulates osteoclastogenesis via targeting RANK. *J. Bone Miner. Res.* 29, 338–347. doi: 10.1002/jbmr.2032

Chen, Y., Wang, X., Di, L., Fu, G., Bai, L., Liu, J., et al. (2008). Phospholipase Cγ2 mediates RANKLstimulated lymph node organogenesis and osteoclastogenesis. *J. Biol. Chem.* 283, 29593–29601. doi: 10.1074/jbc.m802493200

Choi, Y., Arron, J. R., and Townsend, M. J. (2009). Promising bone-related therapeutic targets for rheumatoid arthritis. *Nat. Rev. Rheumatol.* 5, 543–548. doi: 10.1038/nrrheum.2009.175

Coleman, R., and Rubens, R. D. (1987). The clinical course of bone metastases from breast cancer. *Br. J. Cancer* 55, 61–66. doi: 10.1038/bjc.1987.13

Cox, T. R., Rumney, R. M. H., Schoof, E. M., Perryman, L., Høye, A. M., Agrawal, A., et al. (2015). The hypoxic cancer secretome induces pre-metastatic bone lesions through lysyl oxidase. *Nature* 522, 106–110. doi: 10.1038/nature14492

Croucher, P. I., McDonald, M. M., and Martin, T. J. (2016). Bone metastasis: the importance of the neighbourhood. *Nat. Rev. Cancer* 16, 373–386. doi: 10.1038/nrc.2016.44

Csete, D., Simon, E., Alatshan, A., Aradi, P., Dobó-Nagy, C., Jakus, Z., et al. (2019). Hematopoietic or osteoclast-specific deletion of Syk leads to increased bone mass in experimental mice. *Front. Immunol.* 10:937. doi: 10.3389/fimmu.2019.00937

Dbouk, H., Khalil, B. D., Wu, H., Shymanets, A., Nürnberg, B., and Backer, J. M. (2013). Characterization of a tumor-associated activating mutation of the p110β PI 3-kinase. *PLoS One* 8:e63833. doi: 10.1371/journal.pone.0063833

De Vries, T., Schoenmaker, T., Aerts, D., Grevers, L. C., Souza, P. P., Nazmi, K., et al. (2015). M-CSF priming of osteoclast precursors can cause osteoclastogenesis-insensitivity, which can be prevented and overcome on bone. *J. Cell Physiol.* 230, 210–225. doi: 10.1002/jcp.24702

de Vries, T. J., Mullender, M. G., van Duin, M. A., Semeins, C. M., James, N., Green, T. P., et al. (2009). The Src inhibitor AZD0530 reversibly inhibits the formation and activity of human osteoclasts. *Mol. Cancer Res.* 7, 476–488. doi: 10.1158/1541-7786.mcr-08-0219

Decker, C. E., Yang, Z., Rimer, R., Park-Min, K. H., Macaubas, C., Mellins, E. D., et al. (2015). Tmem178 acts in a novel negative feedback loop targeting NFATc1 to regulate bone mass. *Proc. Natl. Acad. Sci. U.S.A.* 112, 15654–15659. doi: 10.1073/pnas.1511285112

Demers, M. J., Thibodeau, S., Noel, D., Fujita, N., Tsuruo, T., Gauthier, R., et al. (2009). Intestinal epithelial cancer cell anoikis resistance: EGFR-mediated sustained activation of Src overrides Fak-dependent signaling to MEK/Erk and/or PI3-K/Akt-1. *J. Cell Biochem.* 107, 639–654. doi: 10.1002/jcb.22131

Di Cristofano, A., and Pandolfi, P. P. (2000). The multiple roles of PTEN in tumor suppression. *Cell* 100, 387–390. doi: 10.1016/s0092-8674(00)80674-1

Edwards, C., and Mundy, G. R. (2008). Eph receptors and ephrin signaling pathways: a role in bone homeostasis. *Int. J. Med. Sci.* 5, 263–272. doi: 10.7150/ijms.5.263

Edwards, J. C., Cohen, C., Xu, W., and Schlesinger, P. H. (2006). c-Src control of chloride channel support for osteoclast HCl transport and bone resorption. *J. Biol. Chem.* 281, 28011–28022. doi: 10.1074/jbc.m605865200

Ell, B., Mercatali, L., Ibrahim, T., Campbell, N., Schwarzenbach, H., Pantel, K., et al. (2013). Tumor-induced osteoclast miRNA changes as regulators and biomarkers of osteolytic bone metastasis. *Cancer Cell* 24, 542–556. doi: 10.1016/j.ccr.2013.09.008

Epple, H., Cremasco, V., Zhang, K., Mao, D., Longmore, G. D., and Faccio, R. (2008). Phospholipase Cγ2 modulates integrin signaling in the osteoclast by affecting the localization and activation of Src kinase. *Mol. Cell Biol.* 28, 3610–3622. doi: 10.1128/mcb.00259-08

Faccio, R. (2011). Immune regulation of the tumor/bone vicious cycle. *Ann. N. Y. Acad. Sci.* 1237, 71–78. doi: 10.1111/j.1749-6632.2011.06244.x

Faccio, R., Takeshita, S., Zallone, A., Ross, F. P., and Teitelbaum, S. L. (2003). c-Fms and the alphavbeta3 integrin collaborate during osteoclast differentiation. *J. Clin. Invest.* 115, 749–758. doi: 10.1172/jci200316924

Faccio, R., Teitelbaum, S. L., Fujikawa, K., Chappel, J., Zallone, A., Tybulewicz, V. L., et al. (2005). Vav3 regulates osteoclast function and bone mass. *Nat. Med.* 11, 284–290. doi: 10.1038/nm1194

Farrugia, A. N., Atkins, G. J., To, L. B., Pan, B., Horvath, N., Kostakis, P., et al. (2003). Receptor activator of nuclear factor-kappaB ligand expression by human myeloma cells mediates osteoclast formation in vitro and correlates with bone destruction in vivo. *Cancer Res.* 63, 5438–5445.

Feng, L., Reynisdóttir, I., and Reynisson, J. (2012). The effect of PLC-γ2 inhibitors on the growth of human tumour cells. *Eur. J. Med. Chem.* 54, 463–469. doi: 10.1016/j.ejmech.2012.05.029

Firestein, G. S. (2003). Evolving concepts of rheumatoid arthritis. *Nature* 423, 356–361. doi: 10.1038/nature01661

Fodor, S., Jakus, Z., and Mócsai, A. (2006). ITAM-based signaling beyond the adaptive immune response. *Immunol. Lett.* 104, 29–37. doi: 10.1016/j.imlet.2005.11.001

Franceschetti, T., Kessler, C. B., Lee, S. K., and Delany, A. M. (2013). miR-29 promotes murine osteoclastogenesis by regulating osteoclast commitment and migration. *J. Biol. Chem.* 288, 33347–33360. doi: 10.1074/jbc.m113.484568

Frisch, S. M., and Screaton, R. A. (2001). Anoikis mechanisms. *Curr. Opin. Cell Biol.* 13, 555–562. doi: 10.1016/s0955-0674(00)00251-9

Frisch, S. M., Vuori, K., Ruoslahti, E., and Chan-Hui, P. Y. (1996). Control of adhesion-dependent cell survival by focal adhesion kinase. *J. Cell Biol.* 134, 793–799. doi: 10.1083/jcb.134.3.793

Fruman, D., and Rommel, C. (2014). PI3K and cancer: lessons, challenges and opportunities. *Nat. Rev. Drug Discov.* 13, 140–156. doi: 10.1038/nrd4204

Furuyama, N., and Fujisawa, Y. (2000). Regulation of collagenolytic protease secretion through c-Src in osteoclasts. *Biochem. Biophys. Res. Commun.* 272, 116–124. doi: 10.1006/bbrc.2000.2698

Gabrilovich, D. I., and Nagaraj, S. (2009). Myeloid-derived suppressor cells as regulators of the immune system. *Nat. Rev. Immunol.* 9, 162–174. doi: 10.1038/nri2506

Gabrilovich, D. I., Ostrand-Rosenberg, S., and Bronte, V. (2012). Coordinated regulation of myeloid cells by tumours. *Nat. Rev. Immunol.* 12, 253–268. doi: 10.1038/nri3175

Giuliani, N., Bataille, R., Mancini, C., Lazzaretti, M., and Barille, S. (2001). Myeloma cells induce imbalance in the osteoprotegerin/osteoprotegerin ligand system in the human bone marrow environment. *Blood* 98, 3527–3533. doi: 10.1182/blood.v98.13.3527

Grimaud, E., Soubigou, L., Couillaud, S., Coipeau, P., Moreau, A., Passuti, N., et al. (2003). Receptor activator of nuclear factor kappaB ligand (RANKL)/osteoprotegerin (OPG) ratio is increased in severe osteolysis. *Am. J. Pathol.* 163, 2021–2031. doi: 10.1016/s0002-9440(10)63560-2

Győri, D., Csete, D., Benkő, S., Kulkarni, S., Mandl, P., Dobó-Nagy, C., et al. (2014). The phosphoinositide 3-kinase isoform PI3Kβ regulates osteoclast-mediated bone resorption in humans and mice. *Arthritis Rheumatol.* 66, 2210–2221. doi: 10.1002/art.38660

Győri, D., Lim, E. L., Grant, F. M., Spensberger, D., Roychoudhuri, R., Shuttleworth, S. J., et al. (2018). Compensation between CSF1R+ macrophages and Foxp3+ Treg cells drives resistance to tumor immunotherapy. *JCI Insight.* 3:120631.

Győri, D., and Mócsai, A. (2015). *Osteoclasts in Inflammation*. Berlin: Springer, 1–7.

Hall, T. J., Jeker, H., and Schaueblin, M. (1995). Wortmannin, a potent inhibitor of phosphatidylinositol 3-kinase, inhibits osteoclastic bone resorption in vitro. *Calcif. Tissue Int.* 56, 336–338. doi: 10.1007/bf00318056

Hawkins, P. T., Anderson, K. E., Davidson, K., and Stephens, L. R. (2006). Signalling through class I PI3Ks in mammalian cells. *Biochem. Soc. Trans.* 34, 647–662. doi: 10.1042/bst0340647

Hawkins, P. T., and Stephens, L. R. (2015). PI3K signalling in inflammation. *Biochim. Biophys. Acta* 1851, 882–897. doi: 10.1016/j.bbalip.2014.12.006

Hayashi, M., Nakashima, T., Taniguchi, M., Kodama, T., Kumanogoh, A., and Takayanagi, H. (2012). Osteoprotection by semaphorin 3A. *Nature* 485, 69–74. doi: 10.1038/nature11000

Hernandez, R. K., Wade, S. W., Reich, A., Pirolli, M., Liede, A., and Lyman, G. H. (2018). Incidence of bone metastases in patients with solid tumors: analysis of oncology electronic medical records in the United States. *BMC Cancer* 18:44. doi: 10.1186/s12885-017-3922-0

Hill, K., Kalifa, S., Das, J. R., Bhatti, T., Gay, M., Williams, D., et al. (2010). The role of PI 3-kinase p110beta in AKT signally, cell survival, and proliferation in human prostate cancer cells. *Prostate* 70, 755–764.

Hiraoka, K., Zenmyo, M., Watari, K., Iguchi, H., Fotovati, A., Kimura, Y. N., et al. (2008). Inhibition of bone and muscle metastases of lung cancer cells by a decrease in the number of monocytes/macrophages. *Cancer Sci.* 99, 1595–1602. doi: 10.1111/j.1349-7006.2008.00880.x

Horne, W. C., Neff, L., Chatterjee, D., Lomri, A., Levy, J. B., and Baron, R. (1992). Osteoclasts express high levels of pp60c-src in association with intracellular membranes. *J. Cell Biol.* 119, 1003–1013. doi: 10.1083/jcb.119.4.1003

Humphrey, M. B., Ogasawara, K., Yao, W., Spusta, S. C., Daws, M. R., Lane, N. E., et al. (2004). The signaling adapter protein DAP12 regulates multinucleation during osteoclast development. *J. Bone Miner. Res.* 19, 224–234. doi: 10.1359/jbmr.0301234

Hynes, R. O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 69, 11–25. doi: 10.1016/0092-8674(92)90115-s

Hynes, R. O. (2002). Integrins: bidirectional, allosteric signaling machines. *Cell* 110, 673–687.

Insogna, K. L., Sahni, M., Grey, A. B., Tanaka, S., Horne, W. C., Neff, L., et al. (1997). Colony-stimulating factor-1 induces cytoskeletal reorganization and c-src-dependent tyrosine phosphorylation of selected cellular proteins in rodent osteoclasts. *J. Clin. Invest.* 100, 2476–2485. doi: 10.1172/jci119790

Jones, D. H., Nakashima, T., Sanchez, O. H., Kozieradzki, I., Komarova, S. V., Sarosi, I., et al. (2006). Regulation of cancer cell migration and bone metastasis by RANKL. *Nature* 440, 692–696.

Joyce-Shaikh, B., Bigler, M. E., Chao, C. C., Murphy, E. E., Blumenschein, W. M., Adamopoulos, I. E., et al. (2010). Myeloid DAP12-associating lectin (MDL)-1 regulates synovial inflammation and bone erosion associated with autoimmune arthritis. *J. Exp. Med.* 207, 579–589. doi: 10.1084/jem.20090516

Kacinski, B. M. (1995). CSF-1 and its receptor in ovarian, endometrial and breast cancer. *Ann. Med.* 27, 79–85. doi: 10.3109/07853899509031941

Kagiya, T. (2015). MicroRNAs and osteolytic bone metastasis: the roles of microRNAs in tumor-induced osteoclast differentiation. *J. Clin. Med.* 4, 1741–1752. doi: 10.3390/jcm4091741

Kakonen, S. M., Selander, K. S., Chirgwin, J. M., Yin, J. J., Burns, S., Rankin, W. A., et al. (2002). Transforming growth factor-beta stimulates parathyroid hormone-related protein and osteolytic metastases via Smad and mitogen-activated protein kinase signaling pathways. *J. Biol. Chem.* 277, 24571–24578. doi: 10.1074/jbc.m202561200

Kameda, Y., Takahata, M., Komatsu, M., Mikuni, S., Hatakeyama, S., Shimizu, T., et al. (2013). Siglec-15 regulates osteoclast differentiation by modulating RANKL-induced phosphatidylinositol 3-kinase/Akt and Erk pathways in association with signaling Adaptor DAP12. *J. Bone Miner. Res.* 28, 2463–2475. doi: 10.1002/jbmr.1989

Kearns, A., Khosla, S., and Kostenuik, P. J. (2008). Receptor activator of nuclear factor kappaB ligand and osteoprotegerin regulation of bone remodeling in health and disease. *Endocr. Rev.* 29, 155–192. doi: 10.1210/er.2007-0014

Kelchtermans, H., Geboes, L., Mitera, T., Huskens, D., Leclercq, G., and Matthys, P. (2009). Activated CD4+CD25+ regulatory T cells inhibit osteoclastogenesis and collageninduced arthritis. *Ann. Rheum. Dis.* 68, 744–750. doi: 10.1136/ard.2007.086066

Kertész, Z., Győri, D., Körmendi, S., Fekete, T., Kis-Tóth, K., Jakus, Z., et al. (2012). Phospholipase Cγ2 is required for basal but not oestrogen deficiency-induced bone resorption. *Eur. J. Clin. Invest.* 42, 49–60. doi: 10.1111/j.1365-2362.2011.02556.x

Kim, H., Kim, T., Jeong, B. C., Cho, I. T., Han, D., Takegahara, N., et al. (2013). Tmem64 modulates calcium signaling during RANKL-mediated osteoclast differentiation. *Cell Metab.* 17, 249–260. doi: 10.1016/j.cmet.2013.01.002

Kim, K., Kim, J. H., Lee, J., Jin, H. M., Kook, H., Kim, K. K., et al. (2007a). MafB negatively regulates RANKL-mediated osteoclast differentiation. *Blood* 109, 3253–3259. doi: 10.1182/blood-2006-09-048249

Kim, N., Kadono, Y., Takami, M., Lee, J., Lee, S. H., Okada, F., et al. (2005). Osteoclast differentiation independent of the TRANCE-RANK-TRAF6 axis. *J. Exp. Med.* 202, 589–595. doi: 10.1084/jem.20050978

Kim, Y., Lee, C. K., Nah, S. S., Mun, S. H., Yoo, B., and Moon, H. B. (2007b). Human CD4+CD25+ regulatory T cells inhibit the differentiation of osteoclasts from peripheral blood mononuclear cells. *Biochem. Biophys. Res. Commun.* 357, 1046–1052. doi: 10.1016/j.bbrc.2007.04.042

Kitazawa, S., and Kitazawa, R. (2002). RANK ligand is a prerequisite for cancer-associated osteolytic lesions. *J. Pathol.* 198, 228–236. doi: 10.1002/path.1199

Koga, T., Inui, M., Inoue, K., Kim, S., Suematsu, A., Kobayashi, E., et al. (2004). Costimulatory signals mediated by the ITAM motif cooperate with RANKL for bone homeostasis. *Nature* 428, 758–763. doi: 10.1038/nature02444

Kostenuik, P. J., Singh, G., Suyama, K. L., and Orr, F. W. (1992). A quantitative model for spontaneous bone metastasis: evidence for a mitogenic effect of bone on Walker 256 cancer cells. *Clin. Exp. Metastasis* 10, 403–410. doi: 10.1007/bf00133469

Kuo, P. L., Liao, S. H., Hung, J. Y., Huang, M. S., and Hsu, Y. L. (2013). MicroRNA-33a functions as a bone metastasis suppressor in lung cancer by targeting parathyroid hormone related protein. *Biochim. Biophys. Acta* 1830, 3756–3766. doi: 10.1016/j.bbagen.2013.02.022

Lee, Y., Kim, H. J., Park, C. K., Kim, Y. G., Lee, H. J., Kim, J. Y., et al. (2013). MicroRNA-124 regulates osteoclast differentiation. *Bone* 56, 383–389. doi: 10.1016/j.bone.2013.07.007

Lin, K., Gong, J., Li, C. F., Jang, T. H., Chen, W. L., Chen, H. J., et al. (2012). Vav3-rac1 signaling regulates prostate cancer metastasis with elevated Vav3 expression correlating with prostate cancer progression and posttreatment recurrence. *Cancer Res.* 72, 3000–3009. doi: 10.1158/0008-5472.can-11-2502

Lontos, K., Adamik, J., Tsagianni, A., Galson, D. L., Chirgwin, J. M., and Suvannasankha, A. (2018). The Role of semaphorin 4D in bone remodeling and cancer metastasis. *Front. Endocrinol.* 9:322. doi: 10.3389/fendo.2018.00322

Lorenzo, J., Horowitz, M., and Choi, Y. (2007). Osteoimmunology: interaction of the bone and immune systems. *End. Rev.* 29, 403–440.

Lorenzo, J., Horowitz, M., Choi, Y., and Takayanagi, H. (2010). *Osteoimmunology: Interactions of the Immune and Skeletal Systems*. Cambridge, MA: Academic Press.

Lowe, C., Yoneda, T., Boyce, B. F., Chen, H., Mundy, G. R., and Soriano, P. (1993). Osteopetrosis in Src-deficient mice is due to an autonomous defect of osteoclasts. *Proc. Natl. Acad. Sci. U.S.A.* 90, 4485–4489. doi: 10.1073/pnas.90.10.4485

Mancino, A. T., Klimberg, V. S., Yamamoto, M., Manolagas, S. C., and Abe, E. (2001). Breast cancer increases osteoclastogenesis by secreting M-CSF and upregulating RANKL in stromal cells. *J. Surg. Res.* 100, 18–24. doi: 10.1006/jsre.2001.6204

Mann, M., Barad, O., Agami, R., Geiger, B., and Hornstein, E. (2010). miRNA-based mechanism for the commitment of multipotent progenitors to a single cellular fate. *Proc. Natl. Acad. Sci. U.S.A.* 107, 15804–15809. doi: 10.1073/pnas.0915022107

Mao, D., Epple, H., Uthgenannt, B., Novack, D. V., and Faccio, R. (2006). PLCγ2 regulates osteoclastogenesis via its interaction with ITAM proteins and GAB2. *J. Clin. Invest.* 116, 2869–2879. doi: 10.1172/jci28775

Marton, N., Kovacs, O. T., Baricza, E., Kittel, A., Győri, D., Mocsai, A., et al. (2017). Extracellular vesicles regulate the human osteoclastogenesis: divergent roles in discrete inflammatory arthropathies. *Cell Mol. Life Sci.* 74, 3599–3611. doi: 10.1007/s00018-017-2535-8

McHugh, K. P. (2000). Mice lacking b3 integrins are osteosclerotic because of dysfunctional osteoclasts. *J. Clin. Invest.* 105, 433–440. doi: 10.1172/jci8905

McInnes, I. B., and Schett, G. (2007). Cytokines in the pathogenesis of rheumatoid arthritis. *Nat. Rev. Immunol.* 7, 429–442.

Miyauchi, A., Alvarez, J., Greenfield, E. M., Teti, A., Grano, M., Colucci, S., et al. (1991). Recognition of osteopontin and related peptides by an alpha v beta 3 integrin stimulates immediate cell signals in osteoclasts. *J. Biol. Chem.* 266, 20369–20374.

Miyauchi, Y., Ninomiya, K., Miyamoto, H., Sakamoto, A., Iwasaki, R., Hoshi, H., et al. (2010). The Blimp1-Bcl6 axis is critical to regulate osteoclast

differentiation and bone homeostasis. *J. Exp. Med.* 207, 751–762. doi: 10.1084/jem.20091957

Miyazaki, T., Sanjay, A., Neff, L., Tanaka, S., Horne, W. C., and Baron, R. (2004). Src kinase activity is essential for osteoclast function. *J. Biol. Chem.* 279, 17660–17666. doi: 10.1074/jbc.m311032200

Mócsai, A., Humphrey, M. B., Ziffle, J.A. Van, Hu, Y., Burghardt, A., Spusta, S. C., et al. (2004). The immunomodulatory adapter proteins DAP12 and Fc receptor gamma-chain (FcRγ) regulate development of functional osteoclasts through the Syk tyrosine kinase. *Proc. Natl. Acad. Sci. U.S.A.* 101, 6158–6163. doi: 10.1073/pnas.0401602101

Mócsai, A., Ruland, J., and Tybulewicz, V. L. (2010). The SYK tyrosine kinase: a crucial player in diverse biological functions. *Nat. Rev. Immunol.* 10, 387–402. doi: 10.1038/nri2765

Monteiro, A. C., Leal, A. C., Goncalves-Silva, T., Mercadante, A. C., Kestelman, F., Chaves, S. B., et al. (2013). T cells induce pre-metastatic osteolytic disease and help bone metastases establishment in a mouse model of metastatic breast cancer. *PLoS One* 8:e68171. doi: 10.1371/journal.pone.0068171

Mosser, D. M., and Edwards, J. P. (2008). Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* 8, 958–969. doi: 10.1038/nri2448

Mundy, G. R. (2002). Metastasis: metastasis to bone: causes, consequences and therapeutic opportunities. *Nat. Rev. Cancer* 2, 584–593. doi: 10.1038/nrc867

Munugalavadla, V., Vemula, S., Sims, E. C., Krishnan, S., Chen, S., Yan, J., et al. (2008). The p85alpha subunit of class IA phosphatidylinositol 3-kinase regulates the expression of multiple genes involved in osteoclast maturation and migration. *Mol. Cell Biol.* 28, 7182–7198. doi: 10.1128/mcb.00920-08

Myoui, A., Nishimura, R., Williams, P. J., Hiraga, T., Tamura, D., Michigami, T., et al. (2003). C-SRC tyrosine kinase activity is associated with tumor colonization in bone and lung in an animal model of human breast cancer metastasis. *Cancer Res.* 63, 5028–5033.

Nakamura, I., Takahashi, N., Sasaki, T., Tanaka, S., Udagawa, N., and Murakami, H. (1995). Wortmannin, a specific inhibitor of phosphatidylinositol-3 kinase, blocks osteoclastic bone resorption. *FEBS Lett.* 361, 79–84. doi: 10.1016/0014-5793(95)00153-z

Nakashima, T., and Takayanagi, H. (2009). Osteoimmunology: crosstalk between the immune and bone systems. *J. Clin. Immunol.* 29, 555–567. doi: 10.1007/s10875-009-9316-6

Negishi-Koga, T., Gober, H. J., Sumiya, E., Komatsu, N., Okamoto, K., Sawa, S., et al. (2015). Immune complexes regulate bone metabolism through FcRγ signalling. *Nat. Commun.* 6:6637.

Nishikawa, K., Iwamoto, Y., Kobayashi, Y., Katsuoka, F., Kawaguchi, S., Tsujita, T., et al. (2015). DNA methyltransferase 3a regulates osteoclast differentiation by coupling to an S-adenosylmethionine-producing metabolic pathway. *Nat. Med.* 21, 281–287. doi: 10.1038/nm.3774

Nishikawa, K., Nakashima, T., Hayashi, M., Fukunaga, T., Kato, S., Kodama, T., et al. (2010). Blimp1-mediated repression of negative regulators is required for osteoclast differentiation. *Proc. Natl. Acad. Sci. U.S.A.* 107, 3117–3122. doi: 10.1073/pnas.0912779107

Novack, D. V., and Teitelbaum, S. L. (2008). The osteoclast: friend or foe? *Annu. Rev. Pathol.* 3, 457–484.

Oikawa, T., Oyama, M., Kozuka-Hata, H., Uehara, S., Udagawa, N., Saya, H., et al. (2012). Tks5-dependent formation of circumferential podosomes/invadopodia mediates cell-cell fusion. *J. Cell Biol.* 197, 553–568. doi: 10.1083/jcb.201111116

Okamoto, K., and Takayanagi, H. (2011). Regulation of bone by the adaptive immune system in arthritis. *Arthritis Res. Ther.* 13, 219. doi: 10.1186/ar3323

Okkenhaug, K. (2013). Signaling by the phosphoinositide 3-kinase family in immune cells. *Annu. Rev. Immunol.* 31, 675–704. doi: 10.1146/annurev-immunol-032712-095946

Orr, W., Varani, J., Gondex, M. K., Ward, P. A., and Mundy, G. R. (1979). Chemotactic responses of tumor cells to products of resorbing bone. *Science* 203, 176–179. doi: 10.1126/science.569363

Paloneva, J., Mandelin, J., Kiialainen, A., Bohling, T., Prudlo, J., Hakola, P., et al. (2003). DAP12/TREM2 deficiency results in impaired osteoclast differentiation and osteoporotic features. *J. Exp. Med.* 198, 669–675. doi: 10.1084/jem.20030027

Pilkington, M. F., Sims, S. M., and Dixon, S. J. (1998). Wortmannin inhibits spreading and chemotaxis of rat osteoclasts in vitro. *J. Bone Miner. Res.* 13, 688–694. doi: 10.1359/jbmr.1998.13.4.688

Pixley, F. J., and Stanley, E. R. (2004). CSF-1 regulation of the wandering macrophage: complexity in action. *Trends Cell Biol.* 14, 628–638. doi: 10.1016/j.tcb.2004.09.016

Qin, J., Vinogradova, O., and Plow, E. F. (2004). Integrin bidirectional signaling: a molecular view. *PLoS Biol.* 2:e169. doi: 10.1371/journal.pbio.0020169

Ries, C., Hoves, S., Cannarile, M. A., and Rüttinger, D. (2015). CSF-1/CSF-1R targeting agents in clinical development for cancer therapy. *Curr. Opin. Pharmacol.* 23, 45–51. doi: 10.1016/j.coph.2015.05.008

Roodman, G. D., and Dougall, W. C. (2008). RANK ligand as a therapeutic target for bone metastases and multiple myeloma. *Cancer Treat. Rev.* 34, 92–101. doi: 10.1016/j.ctrv.2007.09.002

Ross, F. P., Chappel, J., Alvarez, J. I., Sander, D., Butler, W. T., Farach-Carson, M. C., et al. (1993). Interactions between the bone matrix proteins osteopontin and bone sialoprotein and the osteoclast integrin alpha v beta 3 potentiate bone resorption. *J. Biol. Chem.* 268, 9901–9907.

Ross, F. P., and Teitelbaum, S. L. (2005). αvβ3 and macrophage colony-stimulating factor: partners in osteoclast biology. *Immunol. Rev.* 208, 88–105. doi: 10.1111/j.0105-2896.2005.00331.x

Samuels, Y., Diaz, L. A., Schmidt-Kittler, O., Cummins, J. M., Delong, L., Cheong, I., et al. (2005). Mutant PIK3CA promotes cell growth and invasion of human cancer cells. *Cancer Cell* 7, 561–573. doi: 10.1016/j.ccr.2005.05.014

Sasaki, T., Irie-Sasaki, J., Jones, R. G., Oliveira-dos-Santos, A. J., Stanford, W. L., Bolon, B., et al. (2000). Function of PI3Kgamma in thymocyte development, T cell activation, and neutrophil migration. *Science* 287, 1040–1046. doi: 10.1126/science.287.5455.1040

Sato, K., Suematsu, A., Nakashima, T., Takemoto-Kimura, S., Aoki, K., Morishita, Y., et al. (2006a). Regulation of osteoclast differentiation and function by the CaMK-CREB pathway. *Nat. Med.* 12, 1410–1416. doi: 10.1038/nm1515

Sato, K., Suematsu, A., Okamoto, K., Yamaguchi, A., Morishita, Y., Kadono, Y., et al. (2006b). Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. *J. Exp. Med.* 203, 2673–2682. doi: 10.1084/jem.20061775

Sato, M., Bryant, H. U., Dodge, J. A., Davis, H., Matter, W. F., and Vlahos, C. J. (1996). Effects of wortmannin analogs on bone in vitro and in vivo. *J. Pharmacol. Exp. Ther.* 277, 543–550.

Sawant, A., Deshane, J., Jules, J., Lee, C. M., Harris, B. A., Feng, X., et al. (2013). Myeloid-derived suppressor cells function as novel osteoclast progenitors enhancing bone loss in breast cancer. *Cancer Res.* 73, 672–682. doi: 10.1158/0008-5472.can-12-2202

Sawyer, C., Sturge, J., Bennett, D. C., O'Hare, M. J., Allen, W. E., Bain, J., et al. (2003). Regulation of breast cancer cell chemotaxis by the phosphoinositide 3-kinase p110delta. *Cancer Res.* 63, 1667–1675.

Scheid, M. P., and Woodgett, J. R. (2001). PKB/Akt: functional insights from genetic models. *Nat. Rev. Mol. Cell Biol.* 2, 760–768. doi: 10.1038/35096067

Schett, G. (2009). Osteoimmunology in rheumatic diseases. *Arth. Res. Ther.* 11:210. doi: 10.1186/ar2571

Schett, G., and Teitelbaum, S. L. (2009). Osteoclasts and arthritis. *J. Bone Miner. Res.* 24, 1142–1146.

Scholl, S. M., Pallud, C., Beuvon, F., Hacene, K., Stanley, E. R., Rohrschneider, L. R., et al. (1994). Anti-colony-stimulating factor-1 antibody staining in primary breast adenocarcinomas correlates with marked inflammatory cell infiltrates and prognosis. *J. Natl. Cancer Inst.* 86, 120–126. doi: 10.1093/jnci/86.2.120

Schreiber, R. D., Old, L. J., and Smyth, M. J. (2011). Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* 331, 1565–1570. doi: 10.1126/science.1203486

Schwartz, M. A., Schaller, M. D., and Ginsberg, M. H. (1995). Integrins: emerging paradigms of signal transduction. *Annu. Rev. Cell Dev. Biol.* 11, 549–599. doi: 10.1146/annurev.cb.11.110195.003001

Sezer, O., Heider, U., Zavrski, I., Kuhne, C. A., and Hofbauer, L. C. (2003). RANK ligand and osteoprotegerin in myeloma bone disease. *Blood* 101, 2094–2098. doi: 10.1182/blood-2002-09-2684

Shabo, I., Olsson, H., Stål, O., and Svanvik, J. (2013). Breast cancer expression of DAP12 is associated with skeletal and liver metastases and poor survival. *Clin. Breast. Cancer* 13, 371–377. doi: 10.1016/j.clbc.2013.05.003

Shain, K. H., Landowski, T. H., and Dalton, W. S. (2002). Adhesion-mediated intracellular redistribution of c-Fas-associated death domain-like IL-1-converting enzyme-like inhibitory protein-long confers resistance to

CD95-induced apoptosis in hematopoietic cancer cell lines. *J. Immunol.* 168, 2544–2553. doi: 10.4049/jimmunol.168.5.2544

Shattil, S. J., Kim, C., and Ginsberg, M. H. (2010). The final steps of integrin activation: the end game. *Nat. Rev. Mol. Cell Biol.* 11, 288–300. doi: 10.1038/nrm2871

Shinohara, M., Nakamura, M., Masuda, H., Hirose, J., Kadono, Y., Iwasawa, M., et al. (2012). Class IA phosphatidylinositol 3-kinase regulates osteoclastic bone resorption through protein kinase B-mediated vesicle transport. *J. Bone Miner. Res.* 27, 2464–2475. doi: 10.1002/jbmr.1703

Shugg, R. P., Thomson, A., Tanabe, N., Kashishian, A., Steiner, B. H., Puri, K. D., et al. (2013). Effects of isoform-selective phosphatidylinositol 3-kinase inhibitors on osteoclasts: actions on cytoskeletal organization, survival, and resorption. *J. Biol. Chem.* 288, 35346–35357. doi: 10.1074/jbc.m113.507525

Simonet, W. S., Lacey, D. L., Dunstan, C. R., Kelley, M., Chang, M. S., Luthy, R., et al. (1997). Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* 89, 309–319. doi: 10.1016/s0092-8674(00)80209-3

Simpson, K. J., Selfors, L. M., Bui, J., Reynolds, A., Leake, D., Khvorova, A., et al. (2008). Identification of genes that regulate epithelial cell migration using an siRNA screening approach. *Nat. Cell. Biol.* 10, 1027–1038. doi: 10.1038/ncb1762

Sloan, E., Pouliot, N., Stanley, K. L., Chia, J., Moseley, J. M., Hards, D. K., et al. (2006). Tumor-specific expression of alphavbeta3 integrin promotes spontaneous metastasis of breast cancer to bone. *Breast Cancer Res.* 8:R20.

Smith, H. O., Anderson, P. S., Kuo, D. Y. K., Goldberg, G. L., DeVictoria, C. L., Boocock, C. A., et al. (1995). The role of colony-stimulating factor 1 and its receptor in the etiopathogenesis of endometrial adenocarcinoma. *Clin. Cancer Res.* 1, 313–325.

Soriano, P., Montgomery, C., Geske, R., and Bradley, A. (1991). Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. *Cell* 64, 693–702. doi: 10.1016/0092-8674(91)90499-o

Stanley, E. R., Guilbert, L. T., Tushinski, R. J., and Bartelmez, S. H. (1983). CSF-1 A mononuclear phagocyte lineage-specific hemopoietic growth factor. *J. Cell Biochem.* 21, 151–159. doi: 10.1002/jcb.240210206

Stiffel, V., Amoui, M., Sheng, M. H., Mohan, S., and Lau, K. H. (2014). EphA4 receptor is a novel negative regulator of osteoclast activity. *J. Bone Miner. Res.* 29, 804–819. doi: 10.1002/jbmr.2084

Sugatani, T., and Hruska, K. A. (2007). MicroRNA-223 is a key factor in osteoclast differentiation. *J. Cell Biochem.* 101, 996–999. doi: 10.1002/jcb.21335

Suva, L. J., Winslow, G. A., Wettenhall, R. E., Hammonds, R. G., Moseley, J. M., Diefenbach-Jagger, H., et al. (1987). A parathyroid hormone-related protein implicated in malignant hypercalcemia: cloning and expression. *Science* 237, 893–896. doi: 10.1126/science.3616618

Takayanagi, H. (2007). Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. *Nat. Rev. Immunol.* 7, 292–304. doi: 10.1038/nri2062

Takayanagi, H. (2010). New immune connections in osteoclast formation. *Ann. N. Y. Acad. Sci.* 1192, 117–123. doi: 10.1111/j.1749-6632.2009.05303.x

Takegahara, N., Takamatsu, H., Toyofuku, T., Tsujimura, T., Okuno, T., Yukawa, K., et al. (2006). Plexin-A1 and its interaction with DAP12 in immune responses and bone homeostasis. *Nat. Cell Biol.* 8, 615–622. doi: 10.1038/ncb1416

Tan, W., Zhang, W., Strasner, A., Grivennikov, S., Cheng, J. Q., Hoffman, R. M., et al. (2011). Tumour-infiltrating regulatory T cells stimulate mammary cancer metastasis through RANKL–RANK signalling. *Nature* 470, 548–553. doi: 10.1038/nature09707

Tanaka, S. (2017). RANKL-independent osteoclastogenesis: a long-standing controversy. *J. Bone Miner. Res.* 32, 431–433. doi: 10.1002/jbmr.3092

Tang, R. P., Kackinski, B., Validire, P., Beuvon, F., Sastre, X., Benoit, P., et al. (1990). Oncogene amplification correlates with dense lymphocyte infiltration in human breast cancers: a role for hematopoietic growth factor release by tumor cells? *J. Cell Biochem.* 44, 189–198. doi: 10.1002/jcb.240440307

Teitelbaum, S. L. (2000). Bone resorption by osteoclasts. *Science* 289, 1504–1508. doi: 10.1126/science.289.5484.1504

Teitelbaum, S. L., and Ross, F. P. (2003). Genetic regulation of osteoclast development and function. *Nat. Rev. Genet.* 4, 638–649. doi: 10.1038/nrg1122

Thomas, R. J., Guise, T. A., Yin, J. J., Elliott, J., Horwood, N. J., Martin, T. J., et al. (1999). Breast cancer cells interact with osteoblasts to support osteoclast formation. *Endocrinology* 140, 4451–4458. doi: 10.1210/endo.140.10.7037

Thomas, S. M., and Brugge, J. S. (1997). Cellular functions regulated by Src family kinases. *Annu. Rev. Cell Dev. Biol.* 13, 513–609. doi: 10.1146/annurev.cellbio.13.1.513

Thorpe, L., Yuzugullu, H., and Zhao, J. J. (2015). PI3K in cancer: divergent roles of isoforms, modes of activation and therapeutic targeting. *Nat. Rev. Cancer* 15, 7–24. doi: 10.1038/nrc3860

Tsuji-Takechi, K., Negishi-Koga, T., Sumiya, E., Kukita, A., Kato, S., Maeda, T., et al. (2012). Stage-specific functions of leukemia/lymphoma-related factor (LRF) in the transcriptional control of osteoclast development. *Proc. Natl. Acad. Sci. U.S.A.* 109, 2561–2566. doi: 10.1073/pnas.1116042109

Tsukasaki, M., Hamada, K., Okamoto, K., Nagashima, K., Terashima, A., Komatsu, N., et al. (2017). LOX fails to substitute for RANKL in osteoclastogenesis. *J. Bone Miner. Res.* 32, 434–439. doi: 10.1002/jbmr.2990

Valadi, H., Ekstrom, K., Bossios, A., Sjostrand, M., Lee, J. J., and Lotvall, J. O. (2007). Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* 9, 654–659. doi: 10.1038/ncb1596

Vanhaesebroeck, B., Guillermet-Guibert, J., Graupera, M., and Bilanges, B. (2010). The emerging mechanisms of isoform-specific PI3K signalling. *Nat. Rev. Mol. Cell Biol.* 11, 329–341. doi: 10.1038/nrm2882

Vanhaesebroeck, B., Stephens, L. R., and Hawkins, P. T. (2012). PI3K signalling: the path to discovery and understanding. *Nat. Rev. Mol. Cell Biol.* 13, 195–203. doi: 10.1038/nrm3290

Vivanco, I., and Sawyers, C. L. (2002). The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat. Rev. Cancer* 2, 489–501. doi: 10.1038/nrc839

Weilbaecher, K. N., Guise, T. A., and McCauley, L. K. (2011). Cancer to bone: a fatal attraction. *Nat. Rev. Cancer* 11, 411–425. doi: 10.1038/nrc3055

Wiktor-Jedrzejczak, W., Bartocci, A., Ferrante, A. W. Jr., Ahmed-Ansari, A., Sell, K. W., Pollard, J. W., et al. (1990). Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse. *Proc. Natl. Acad. Sci. U.S.A.* 87, 4828–4832. doi: 10.1073/pnas.87.12.4828

Wong, B., Rho, J., Arron, J., Robinson, E., Orlinick, J., Chao, M., et al. (1997). TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. *J. Biol. Chem.* 272, 25190–25194. doi: 10.1074/jbc.272.40.25190

Yang, Y. M., Kim, M. S., Son, A., Hong, J. H., Kim, K. H., Seo, J. T., et al. (2009). Alteration of RANKL-induced osteoclastogenesis in primary cultured osteoclasts from SERCA2+/- mice. *J. Bone Miner. Res.* 24, 1763–1769. doi: 10.1359/jbmr.090420

Yao, Y., Li, H., Chen, J., Xu, W., Yang, G., Bao, Z., et al. (2016). TREM-2 serves as a negative immune regulator through Syk pathway in an IL-10 dependent manner in lung cancer. *Oncotarget* 7, 29620–29634. doi: 10.18632/oncotarget.8813

Yao, Z., Xing, L., Qin, C., Schwarz, E. M., and Boyce, B. F. (2008). Osteoclast precursor interaction with bone matrix induces osteoclast formation directly by an interleukin-1-mediated autocrine mechanism. *J. Biol. Chem.* 283, 9917–9924. doi: 10.1074/jbc.m706415200

Yasui, T., Hirose, J., Tsutsumi, S., Nakamura, K., Aburatani, H., and Tanaka, S. (2011). Epigenetic regulation of osteoclast differentiation: possible involvement of Jmjd3 in the histone demethylation of Nfatc1. *J. Bone. Miner. Res.* 26, 2665–2671. doi: 10.1002/jbmr.464

Yu, Y., Suryo Rahmanto, Y., Shen, Y. A., Ardighieri, L., Davidson, B., Gaillard, S., et al. (2019). Spleen tyrosine kinase activity regulates epidermal growth factor receptor signaling pathway in ovarian cancer. *EBioMedicine* 47, 184–194. doi: 10.1016/j.ebiom.2019.08.055

Zaidi, M. (2007). Skeletal remodeling in health and disease. *Nat. Med.* 13, 791–801. doi: 10.1038/nm1593

Zaiss, M., Axmann, R., Zwerina, J., Polzer, K., Gückel, E., Skapenko, A., et al. (2007). Treg cells suppress osteoclast formation: a new link between the immune system and bone. *Arthritis Rheum.* 56, 4104–4112. doi: 10.1002/art.23138

Zhang, K., Kim, S., Cremasco, V., Hirbe, A. C., Collins, L., Piwnica-Worms, D., et al. (2011). CD8+ T cells regulate bone tumor burden independent of osteoclast resorption. *Cancer Res.* 71, 4799–4808. doi: 10.1158/0008-5472.can-10-3922

Zhang, Q. W., Liu, L., Gong, C. Y., Shi, H. S., Zeng, Y. H., Wang, X. Z., et al. (2012). Prognostic significance of tumor-associated macrophages in solid tumor: a meta-analysis of the literature. *PLoS One* 7:e50946. doi: 10.1371/journal.pone.0050946

Zhang, Z., Baron, R., and Horne, W. C. (2000). Integrin engagement, the actin cytoskeleton, and c-Src are required for the calcitonin-induced tyrosine phosphorylation of paxillin and HEF1, but not for calcitonin-induced Erk1/2 phosphorylation. *J. Biol. Chem.* 275, 37219–37223. doi: 10.1074/jbc.m001818200

Zhao, B., Grimes, S. N., Li, S., Hu, X., and Ivashkiv, L. B. (2012). TNF-induced osteoclastogenesis and inflammatory bone resorption are inhibited by transcription factor RBP-J. *J. Exp. Med.* 209, 319–334. doi: 10.1084/jem.20111566

Zhao, B., Takami, M., Yamada, A., Wang, X., Koga, T., Hu, X., et al. (2009). Interferon regulatory factor-8 regulates bone metabolism by suppressing osteoclastogenesis. *Nat. Med.* 15, 1066–1071.

Zhao, Y., Bachelier, R., Treilleux, I., Pujuguet, P., Peyruchaud, O., Baron, R., et al. (2007). Tumor alphavbeta3 integrin is a therapeutic target for breast cancer bone metastases. *Cancer Res.* 67, 5821–5830.

Zhuang, J., Zhang, J., Lwin, S. T., Edwards, J. R., Edwards, C. M., Mundy, G. R., et al. (2012). Osteoclasts in multiple myeloma are derived from Gr-1+CD11b+myeloid-derived suppressor cells. *PLoS One* 7:e48871. doi: 10.1371/journal.pone.0048871

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Dual-mTOR Inhibitor Rapalink-1 Reduces Prostate Cancer Patient-Derived Xenograft Growth and Alters Tumor Heterogeneity

*Federico La Manna[1,2], Marta De Menna[1], Nikhil Patel[3], Sofia Karkampouna[1], Maria Rosaria De Filippo[1,4], Irena Klima[1], Peter Kloen[5], Lijkele Beimers[6], George N. Thalmann[7], Rob C. M. Pelger[2], Estela Jacinto[3] and Marianna Kruithof-de Julio[1,7]**

[1] Department for BioMedical Research, Urology Research Laboratory, University of Bern, Bern, Switzerland, [2] Department of Urology, Leiden University Medical Center, Leiden, Netherlands, [3] Department of Biochemistry and Molecular Biology, Robert Wood Johnson Medical School, Rutgers, The State University of New Jersey, Piscataway, NJ, United States, [4] Institute of Pathology and Medical Genetics, University Hospital Basel, University of Basel, Basel, Switzerland, [5] Department of Orthopedic Trauma Surgery, Academic Medical Center, Amsterdam, Netherlands, [6] Department of Orthopedic Surgery, MC Slotervaart, Amsterdam, Netherlands, [7] Department of Urology, Inselspital, Bern University Hospital, Bern, Switzerland

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***Correspondence:**
Marianna Kruithof-de Julio
marianna.kruithofdejulio@dbmr.unibe.ch

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Bone metastasis is the leading cause of prostate cancer (PCa) mortality, frequently marking the progression to castration-resistant PCa. Dysregulation of the androgen receptor pathway is a common feature of castration-resistant PCa, frequently appearing in association with mTOR pathway deregulations. Advanced PCa is also characterized by increased tumor heterogeneity and cancer stem cell (CSC) frequency. CSC-targeted therapy is currently being explored in advanced PCa, with the aim of reducing cancer clonal divergence and preventing disease progression. In this study, we compared the molecular pathways enriched in a set of bone metastasis from breast and prostate cancer from snap-frozen tissue. To further model PCa drug resistance mechanisms, we used two patient-derived xenografts (PDX) models of bone-metastatic PCa, BM18, and LAPC9. We developed *in vitro* organoids assay and *ex vivo* tumor slice drug assays to investigate the effects of mTOR- and CSC-targeting compounds. We found that both PDXs could be effectively targeted by treatment with the bivalent mTORC1/2 inhibitor Rapalink-1. Exposure of LAPC9 to Rapalink-1 but not to the CSC-targeting drug disulfiram blocked mTORC1/2 signaling, diminished expression of metabolic enzymes involved in glutamine and lipid metabolism and reduced the fraction of $CD44^{+}$ and $ALDEFluor^{high}$ cells, *in vitro*. Mice treated with Rapalink-1 showed a significantly delayed tumor growth compared to control and cells recovered from the tumors of treated animals showed a marked decrease of CD44 expression. Taken together these results highlight the increased dependence of advanced PCa on the mTOR pathway, supporting the development of a targeted approach for advanced, bone metastatic PCa.

Keywords: bone metastasis, PDX, mTOR, disulfiram, prostate cancer, ALDH

INTRODUCTION

Development of bone metastasis involves 65–75% of breast and prostate cancer patients with advanced, metastatic disease (1) with the axial skeleton as the most common site of bone metastasis (2).

The clinical implications revolving around the development of a bone metastatic disease include the development of skeletal-related events, like pathological fractures, spine chord compression or bone pain and represent a common event in advanced breast and prostate patients, greatly affecting their quality of life (1, 3). Bone metastasis are frequently characterized by a long latency period, characterized by the presence of subclinical micrometastasis in the bone that are difficult to detect and to target. Once symptomatic, bone metastasis is frequently associated with a progressed, highly malignant relapse of the disease (3). Despite its relevance, the study of bone metastasis has been hindered by the difficulty of obtaining high quality specimens from bone lesions (4, 5).

The dependence of prostate tissue on androgen receptor (AR) signaling prompted the development of AR-targeting molecules, like abiraterone and enzalutamide, for the treatment of metastatic castration-resistant PCa (mCRPC). However, prolonged treatment with these types of drugs fosters the molecular evolution of PCa, increasing its propensity to metastasis formation and to overcome castration (6). Therefore, new and alternative approaches are currently being investigated to overcome or limit this clinically relevant behavior. Patient-derived xenografts (PDX) have proven to be highly valuable tools for the development of precision medicine strategies for the study of PCa (7). BM18 and LAPC9 are bone metastatic PCa models with different molecular and histological features, with androgen-dependent and -independent growth, respectively (8, 9). A relevant advantage of using PDX models is the possibility of investigating cancer stem cells (CSC), a widely recognized hypothesis that accounts for the establishment of a low-cycling, drug-resistant subpopulation of cells with tumor re-growth potential (10–12).

In prostate cancer, CSC have been identified by different parameters, including surface marker expression, subpopulation-specific stainings and functional assays, with various degrees of overlap between the different methods (13, 14).

The link between aldehyde dehydrogenase (ALDH) activity, cell stemness and self-renewal potential, initially found to detect leukemia tumor-initiating cells, was then confirmed also in PCa where it associates with a potentially clinically relevant subpopulation of cells (15–17).

Pharmacological approaches to target the CSC subpopulation of PCa are currently being explored and include disulfiram, a drug used for the treatment of alcohol abuse and currently investigated for its activity against CSC in various tumors, including prostate cancer, glioblastoma and melanoma (11, 18). Multiple mechanisms of the anti-CSC action of disulfiram have been elucidated and include its primary action in the irreversible inhibition of aldehyde dehydrogenase (ALDH), inhibition of ubiquitin-E3 ligase activity, inhibition of epithelial-to-mesenchymal transition (EMT) and increase of reactive oxygen species (ROS) (12, 18). The latter two mechanisms are dependent on the availability of copper as a co-factor, forming equimolar chelation complexes with disulfiram.

The mammalian target of rapamycin (mTOR) is an atypical protein kinase that can participate in two distinct signal transduction complexes, mTORC1 and mTORC2, regulating a plethora of key cellular functions like cell growth, proliferation, survival and metabolism (19, 20). mTORC1 and 2 integrate nutrient availability status with the anabolic needs of the cell. Deregulation of the PI3K/Akt/mTOR pathway in cancer has been well-established and different clinical studies have found an overactivation of this pathway in ∼40% of breast cancers and 50% of primary prostate cancers (21–23). Targeting the AR pathway with androgen blockers increases the activation of the PI3K/Akt/mTOR pathway (24). Conversely, PTEN exerts a regulatory role on the AR, acting both as AR inducer, via an Egr1- and c-Jun-mediated mechanism, and as an AR repressor, by controlling the negative AR regulator Nkx3.1 (25, 26). Recently, AR- and mTOR signaling-dependent metabolic rewiring of PCa cells and during CRPC progression was shown (27). Phase I/II trials on PCa using rapamycin analogs (rapalogs), which inhibit only a subset of mTORC1 functions, revealed clinical inefficacy (28). ATP-competitive mTOR inhibitors, which block both mTORC1 and mTORC2 kinase activity, and dual PI3K/mTOR inhibitors also showed poor efficacy in the clinic due to toxicity (29, 30). Rapalink-1, a bivalent compound that combines the durable effect of rapamycin and dual mTORC1/mTORC2 inhibition, has been developed recently (31). It remains to be examined whether Rapalink-1 would be efficacious for PCa therapy.

The aim of the present study was to determine the impact of the third generation mTOR-inhibiting compound Rapalink-1 using bone-metastatic PCa PDX models *in vitro, ex vivo*, and *in vivo*. We investigated the effects of Rapalink-1 treatment on the CSC compartment and further compared its effects to the CSC-targeting compound disulfiram, exploring the effects of mTOR blockade on the CSC subpopulation.

MATERIALS AND METHODS

Patient Samples

Samples were collected from patients undergoing orthopedic surgery for bone metastasis (prostate cancer, 5 patients; breast cancer, 4 patients) and anonymously analyzed according to the Dutch Medical Research Involving Human Subjects (WMO) act. Samples were either immediately snap-frozen at the time of surgery for further molecular analyses or shipped in Dulbecco modified essential medium (DMEM) supplemented with 1% penicillin/streptomycin (pen/strep) and 1% Glutamax (Thermo Fisher Scientific) for organoids generation.

RNA Isolation and RNA Sequencing

Five mm by 5-mm snap-frozen bone metastasis samples were placed in a tube with 1 ml Tripure reagent (Sigma-Aldrich) and a metallic bead and homogenized with the TissueLyser II (Qiagen) for 2 cycles of 3 min at 30 Hz. In between cycles, samples were incubated at −20°C for 5 min. Manufacturer's protocol was then

followed to extract RNA from the homogenized samples. RNA quality was assessed by Bioanalyzer 2100 (Agilent Technologies) using the Nano kit and following manufacturers protocol. Samples with an "RNA Integrity Number" (RIN) > 7 were further processed for RNA sequencing. Specimens were prepared for RNA sequencing using the "NEBNext Ultra II Directional RNA Library Prep Kit for Illumina" (NEB #E7760S/L) as described previously (32). Briefly, mRNA was isolated from total RNA using the oligo-dT magnetic beads. After fragmentation of the mRNA, a cDNA synthesis was performed. This was used for ligation with the sequencing adapters and PCR amplification of the resulting product. The quality and yield after sample preparation was measured with the Fragment Analyzer. The size of the resulting products was consistent with the expected size distribution (a broad peak between 300 and 500 bp). Clustering and DNA sequencing using the NovaSeq6000 was performed according to manufacturer's protocols. A concentration of 1.1 nM of DNA was used. Image analysis, base calling, and quality check was performed with the Illumina data analysis pipeline RTA3.4.4 and Bcl2fastq v2.20. Sequence reads were aligned using STAR two-pass to the human reference genome GRCh37 (33). RSEM was used to obtain FPKM (fragments per kilobase of exon model per million reads mapped) counts. We removed duplicated gene names when present, keeping the one with highest expression. Gene counts were quantified using the "GeneCounts" option in STAR. Per-gene counts-per-million (CPM) were computed and log2-transformed adding a pseudo-count of 1 to avoid transforming 0. Genes with log2-CPM <1 in more than two samples were removed. Principle component analysis was performed using the top 200 most variable genes. Differential expression analysis was performed using the edgeR package (34). Normalization was performed using the "TMM" (weighted trimmed mean) method and differential expression was assessed using the quasi-likelihood F-test. Genes with FDR <0.05 and >2-fold were considered significantly differentially expressed.

Immunohistochemistry and Histological Stainings

Four-μm thick sections of FFPE blocks were cut, stained for haematoxylin and eosin and mounted with Entellan (Merck-Millipore). For Ki67 and panCK stainings, cut sections were processed for antigen retrieval by pressure cooker for 10 min in citrate buffer at pH 6.0. Sections were allowed to cool and then extensively washed in running water. Endogenous peroxidases were blocked by incubation with 3% H_2O_2 for 15 min at room temperature. Sections were then washed twice with PBS and blocked with a solution of 3% BSA in PBS-Tween 20 0.1% (PBS-T) for 1 h at room temperature then incubated overnight with 100 μl of anti-Ki67 (1:400, rabbit), anti-panCK (1:100, mouse), rabbit IgG or mouse IgG as appropriate, see **Supplementary Information** for a list of the used antibodies. Sections were then washed once with PBS-T and twice with PBS before incubation for 30 min with 100 μl of EnVision anti-rabbit or anti-mouse (Agilent Technologies). Sections were then washed once with PBS-T and twice with PBS and developed in a freshly prepared AEC solution (Dako) until sufficiently developed. Sections were then washed in H_2O and counterstained with hematoxylin before mounting with Entellan. Slides were digitalized with the Pannoramic 250 Flash III slide scanner (3D Histech).

Western Blot

Cells were lysed in RIPA buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM EDTA, 0.2% SDS, 0.5% sodium deoxycholate, 1% Triton-X100) supplemented with protease and phosphatase inhibitors (cOmplete Mini, protease inhibitor cocktail and PhosStop, both by Merck Millipore). Tissue pieces were homogenized with TissueLyser II (Qiagen) for 1 cycle of 2 min at 20 Hz in RIPA buffer, using a metallic bead. Organoids were resuspended in 150 μl of RIPA buffer and homogenized with a 0.3 ml syringe. Homogenized samples were centrifuged for 15 min at >16,000 g at 4°C and supernatant collected. Protein concentrations were measured by Bradford assay and about 10–30 μg of samples were used for SDS-PAGE. Proteins were transferred onto Immobilon-PVDF (Millipore). Blots were incubated with primary antibodies overnight followed by washing with PBS-Tween, see **Supplementary Information** for a list of the used antibodies. Blots were then incubated with either anti-mouse or –rabbit secondary antibody. After washing with PBS-Tween, images were visualized using Supersignal ECL detection kit (ThermoFisher) and captured using Amersham Imager 600 (GE).

Animals Maintenance and *in vivo* Experiment

Animal experiments were conducted according to Bern cantonal guidelines. Mice had unrestricted access to food and fresh water and housed in max 5 animals per cage. For xenograft surgery, nine 5-week old male CB17/SCID mice were anesthetized by subcutaneous injection with a cocktail of medetomidin (Dorbene) 1 mg/kg, midazolam (Dormicum) 10 mg/kg, and fentanyl 0.1 mg/kg. Under sterile hood, two 3 mm long incisions were performed on each side in the scapular region and a small pocket was created by lifting the skin with forceps. Freshly harvested 2 mm^3 tumor pieces were inserted into the pockets, that were closed with resorbable 6-0 suture (Vicryl 6-0, Ethicon). Anesthesia was reversed by subcutaneous injection with atipamezol (Revertor®) 2.5 mg/kg and flumazenil (Anexate®) 0.5 mg/kg, together with buprenorphine (Temgesic) 0.1 mg/kg for analgesia, and sutured wound was disinfected with a iodopovidone solution. Three days post-implantation animals were divided into 2 groups, stratified by weight. Group 1 received 3.5 μl/g of vehicle (20% DMSO, 40% PEG-300 and 40% PBS) i.p. once a week while group 2 received Rapalink-1 (1.5 mg/Kg) resuspended in vehicle, i.p. every 5–7 days. Mouse weight, tumor size and signs of acute toxicities were monitored twice a week, tumor size was tracked by palpation and referred to standardized size beads, to minimize animals' discomfort during the experiment. Mice were euthanized as soon as signs of acute toxicity were detected or when tumor size reached 8 mm.

Organoid Culture

Tissues were collected in basis medium [Advanced D-MEM/F-12 (ThermoFisher Scientific) supplemented with 1 ml Primocin (Invivogen), 1% GlutaMAX and HEPES 10 mM (ThermoFisher Scientific)], finely minced with a scalpel and incubated in 5 mg/ml collagenase type II (Gibco), supplemented with 15 μg/ml DNase I (Sigma-Aldrich) and 10 mM Y-27632, at 37°C for 1–3 h with occasional mixing, until completely digested. Cell suspension was then centrifuged at 400 rcf for 5 min and washed with basis medium. Cell pellet was then incubated at 37°C in 2 ml TripLE Express (ThermoFisher Scientific) for 10 min, pipetting cell suspension every 5 min. Digested cell suspension was passed through a 50 μm-pore size strainer (Celltrics, Sysmex) and washed with basis medium. When required, cells were incubated for 5 min in erythrocytes-lysing buffer to eliminate red blood cells, then washed with basis medium. Cells were counted with trypan blue with an automated cell counter (TC20, Bio-Rad), centrifuged and resuspended in complete prostate cancer organoid medium [see **Supplementary Information** for the complete recipe, reproduced from (35)] at 300,000 cells/ml and seeded in 1.5 ml volume in 6-well ultra-low attachment plates (ULA plates, Corning). Fresh medium was added every 2–3 days until organoids were used for downstream applications. For drug pre-treatment, LAPC9 and BM18 organoids were cultured in 6-well ULA plates in complete PCa medium for 48 h, then medium was replaced with fresh medium containing the target drug at the reported concentration and organoids were cultured for further 48 h before proceeding with downstream analysis.

Drug Assay

Organoids were collected in basis medium and centrifuged for 3 min at 100 rcf, then they were resuspended in TripLE Express and incubated at 37°C with occasional resuspension until completely dissociated. Cell suspension was then washed with basis medium and centrifuged at 300 rcf for 5 min. Cells were resuspended at 175,000 cells/ml in complete PCa organoids medium and seeded in 20 μl volume in a 384-well low-attachment plate, with black walls (Corning). After 48 h, wells with organoids were treated with the appropriate compound, resuspended in 20 μl of PCa organoids medium. The compounds used were Rapalink-1 (10–0.001 μM, ApexBio), rapamycin (10–0.1 μM), everolimus (1–0.1 μM), abiraterone (1 μM, in EtOH), enzalutamide (10 μM), disulfiram (10–0.1 μM, Sigma-Aldrich), DMSO (0.1%), and EtOH (0.1%). Where not stated otherwise, the compounds were from Selleckchem, and were resuspended in DMSO. Wells treated with disulfiram were additionally supplemented with copper-gluconate 1 μM (Sigma-Aldrich). Each condition was assessed in quadruplicates, each experiment was repeated 3 times for the PDX models and 2 times for the patient-derived bone metastasis material.

Ex vivo Tissue Culture

Freshly collected LAPC9 and BM18 tissues were aseptically cut into 1 mm-thick serial slices. Tissue slices were then carefully placed on the membrane of a 0.4 μM pores polypropylene 24-well transwell (ThinCert, Grainer Bio-one International) and cultured on 0.5 ml of DMEM supplemented with 10% FBS, 1% pen/strep and the indicated compound for 5 days at 37°C. Before starting the incubation, the plate with the tissue slices was inserted in a sealed chamber and flushed for 3 min with O_2 (3 L/min). After the 5 days, the tissues were collected and fixed for 2 h in 4% PFA under constant agitation, then washed in PBS, dehydrated and embedded in paraffin.

Flow Cytometry

Single cells from dissociated organoids or from digested tissues were washed in FACS buffer (0.5% BSA, 2 mM EDTA in PBS, pH 7.4). Cells were resuspended in a total of 100 μl of FACS buffer with anti-CD44-APC (1:20, BD Bioscience, clone G515) and incubated for 20 min in the dark at room temperature. Cells were then washed once in FACS buffer before proceeding to ALDEFluor staining, as per manufacturer's indications. Tubes were incubated for 45′ at 37°C. After the incubation, cells were washed in ALDEFluor buffer (AB) and resuspended in 300 μl of AB per tube, supplemented with 5 μg/mL of DAPI and kept on ice until acquisition with a BD LSRII flow cytometer (BD Biosciences).

Data Analysis

Data was analyzed using Prism GraphPad 8. Flowcytometry data was analyzed using FlowJo v. 10.6.2. All samples acquired by flow cytometry were analyzed with technical gates by the identification of the population of interest in a SSC-A/FSC-A dot plot, followed by a doublets-excluding gate in a FSC-H/FSC-A dot plot and by a viability gate for DAPI exclusion in a DAPI-A/FSC-A dot plot. For samples stained with ALDEFluor, a minimum of 100,000 events was acquired, for other samples a minimum of 30,000 was acquired, experiments were run in biological duplicates. Gating for ALDEFluor-high (ALDH-hi) cells was setup in the DEAB-treated, matched control sample using median and robust standard deviation (rSD) of fluorescence according to the following formula:

ALDH-hi threshold = ($FITC_{[Median]}$ of DAPI-negative cells) + (3*$FITC_{[rSD]}$ of DAPI-negative cells)

Ki67 immunohistochemistry was quantified with ImageJ (v1.52p). A macro was developed to semi-automatically segment and quantify nuclei and AEC signal. Staining is reported as fraction of Ki67-positive nuclei over total counted nuclei, quantifying at least 5 fields per condition.

RESULTS

Bone Metastasis From Breast and Prostate Cancer Have Distinctive Molecular Signatures

We investigated the molecular profile of snap-frozen bone metastasis specimens from patients with advanced breast or prostate cancer. For a few samples that were available in sufficient amount, a portion of fresh specimen was fixed and paraffin embedded to perform both a hematoxylin and eosin (HE) histological staining and an immunohistochemistry for cytokeratins (panCK) on cut sections (**Figure 1A**). The detection of cytokeratin-positive cells as well as the overall poorly organized bone structure in the analyzed sections confirmed the presence of epithelial cells in the bone sample and a pathological,

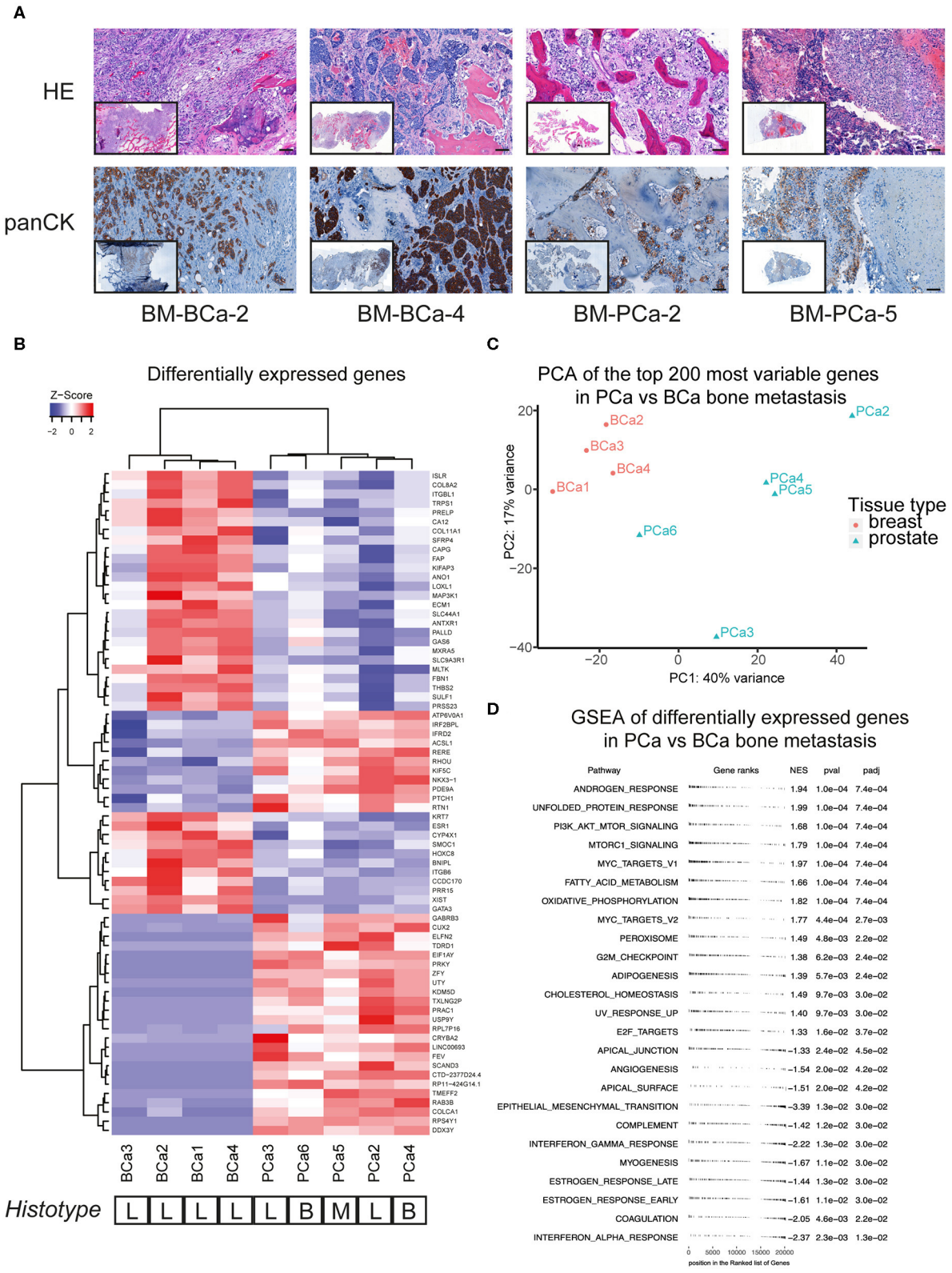


FIGURE 1 | (A) Histological sections of breast and prostate bone metastasis. Sections were stained with hematoxylin and eosin (upper row) and for cytokeratin expression (lower row). Whole-section image included in caption. Scale bar 100 μm. **(B)** Analysis of differentially expressed genes of breast and prostate bone

(Continued)

FIGURE 1 | metastasis, with unsupervised cluster analysis of both samples and genes. The histological type of bone lesion is reported below the heatmap and is referenced as lytic (L), blastic (B), or mixed (M). **(C)** Principal component analysis (PCA) of the top-200 most differentially expressed genes between the BCa and PCa bone metastasis samples included in **(B)**. While all BCa bone metastasis form a defined cluster, PCa bone metastasis show a more scattered profile that does not recapitulate their histological subtype. **(D)** Geneset enrichment analysis (GSEA) of differentially expressed genes shown in **(B)**. Scores > 0 identify genesets enriched in the prostate bone metastasis group, while scores < 0 identify genesets enriched in the breast bone metastasis group. Only significantly enriched genesets are shown.

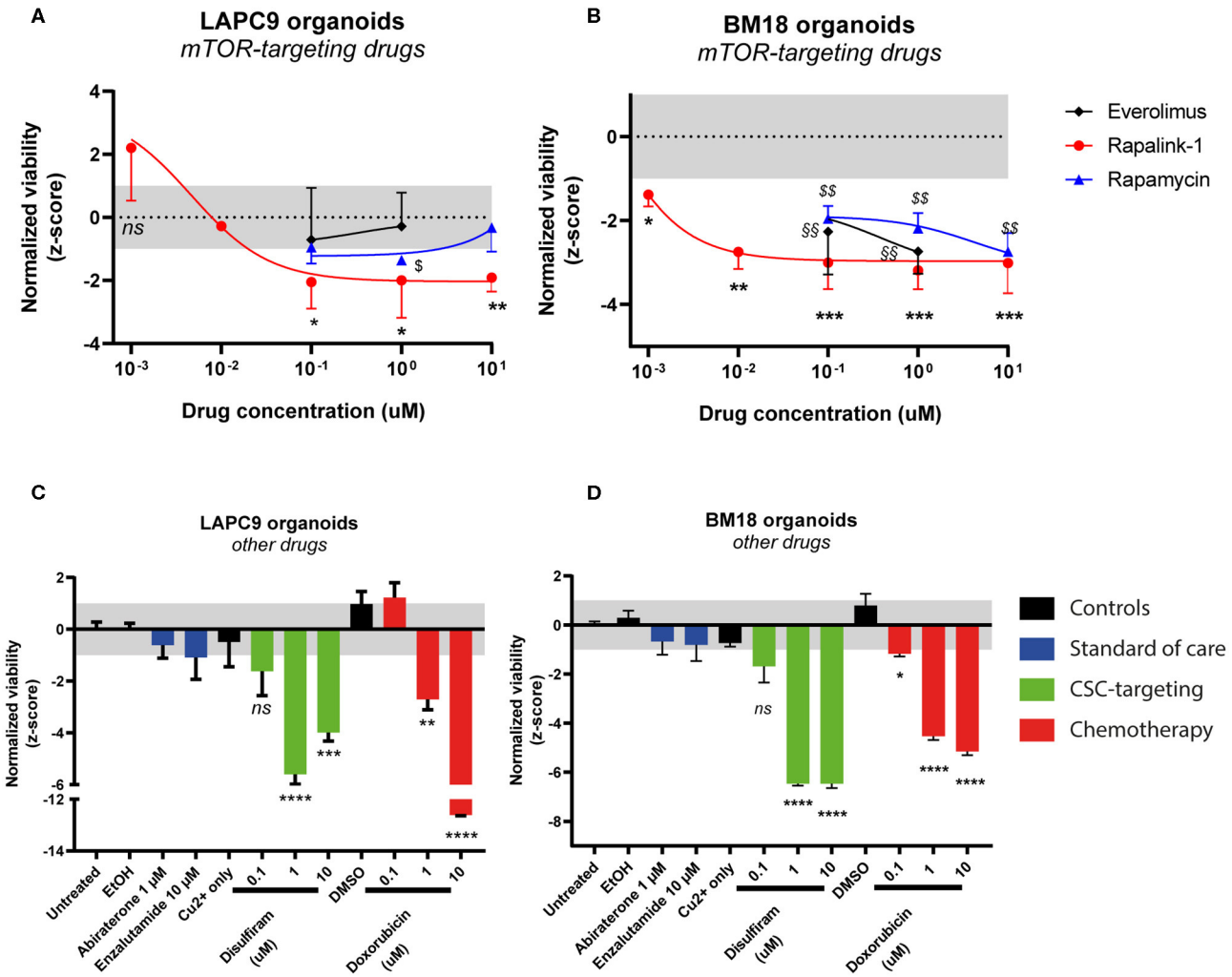


FIGURE 2 | Dose-response curves of LAPC9 **(A)** and BM18 **(B)** PDX organoids to mTOR-targeting drugs rapalink-1 (red circles), rapamycin (blue triangles), and everolimus (black diamonds) after 48 h treatment. Normalized viability values of organoids are plotted against $\log_{10}$ drug concentrations; $N = 2–3$. Viability assay of LAPC9 **(C)** and BM18 **(D)** PDX organoids after treatment for 48 h with other, non mTOR-targeting drugs. The grayed area in plots **(A–D)** corresponds to the expected distribution of the reference condition (untreated). Data were analyzed by one-way ANOVA, treated conditions were compared to vehicle (DMSO, EtOH for abiraterone treatment); $N = 2–3$. *$p < 0.05$; **, §§$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

metastasis-induced bone remodeling process. We performed RNASeq analysis on the bone metastasis specimens, the most differentially expressed genes among the included samples are reported (**Figure 1B**).

The samples formed two subgroups by unsupervised cluster analysis, reflecting the primary cancer of origin. Of note, the bone metastasis samples from prostate cancer did not cluster according to their histotype (lytic, blastic, or mixed lesions), rather by molecular features. The molecular clustering was further investigated by principal component analysis of the top 200 most differentially expressed genes, between the BCa and PCa bone metastasis samples. While all BCa bone metastasis samples formed one cluster, the PCa bone metastasis showed a more scattered distribution, that did not correspond to the histological bone lesion type (**Figure 1C**). Further pathways analysis on the differentially expressed genes in metastatic PCa highlighted

the enrichment of androgen response genes, together with processes linked to lipid metabolism (adipogenesis, cholesterol homeostasis, peroxisome, fatty acid metabolism, **Figure 1D**). Moreover, PCa bone metastasis showed a specific enrichment for the mTOR pathway, compared to BCa bone metastasis, which showed a specific enrichment for inflammatory processes (interferon response, angiogenesis) and for genes involved in epithelial-to-mesenchymal transition.

Dual mTORC1/mTORC2 Blockade and ALDH Inhibition Reduce Advanced PCa Organoids Viability *in vitro*

We investigated the effects of mTOR-targeting drugs rapamycin, everolimus and Rapalink-1, a 3rd generation dual mTORC1/2 inhibitor on BM18 and LAPC9 PDX, *in vitro* on organoids. Drug assays on PDX organoids indicated that both LAPC9 and BM18 organoids viability was significantly reduced when treated with Rapalink-1, with a higher IC_{50} in LAPC9 organoids (0.0046 μM) compared to BM18 organoids (0.0003 μM). LAPC9 organoid viability could not be reduced by everolimus at the tested concentrations and could be significantly reduced by rapamycin only at 1 μM, evidencing on the other hand an average viability at 10 μM. BM18 instead showed significant reduction of organoid viability when treated with either everolimus or rapamycin (**Figures 2A,B**).

We then assessed the effects of the standard of care drugs (abiraterone and enzalutamide) on organoids from both PDX, comparing them to disulfiram and to doxorubicin, this latter used for its efficacy on both PDX models (**Figures 2C,D**). After 48 h drug exposure, none of the standard of care drugs had significant impact on organoid viability. On the other hand, doxorubicin effectively and dose-dependently reduced viability of both LAPC9 and BM18 organoids. Treatment with disulfiram 1–10 μM, in presence of 1 μM copper gluconate, also significantly

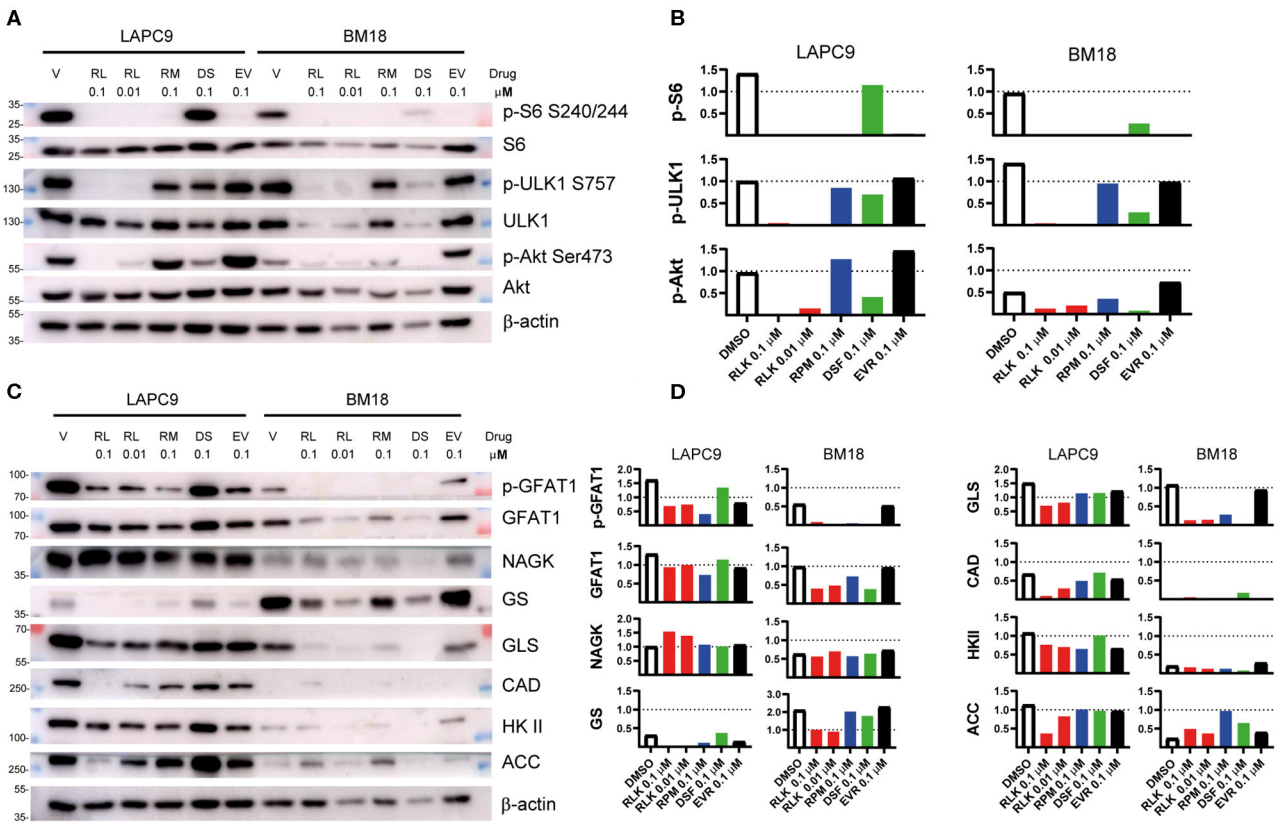


FIGURE 3 | Total lysates of LAPC9 and BM18 organoids treated with rapalink-1 (RL, red bars), rapamycin (RM, blue bars), everolimus (EV, black bars), disulfiram (DS, green bars), or vehicle (V, DMSO, open bars), at the reported concentrations (μM) for 48 h were fractionated by SDS-PAGE followed by Western blotting. Phosphorylated sites or total proteins were detected by immunoblotting using antibodies against the indicated phosphosites or protein. Molecular weight (MW) marker sizes are indicated on the left. Beta-actin was used as loading control. **(A)** The activation status of mTOR was assessed by analyzing the expression of the mTORC1 downstream targets ULK (p-ULK1, S757) and S6 (pS6, S240/244) and of the mTORC2 downstream target Akt (p-Akt, S473). **(B)** Quantification of the phosphosites analyzed in **(A)**. **(C)** The lysates analyzed in **(A)** were further assayed for the activation of the hexosamine biosynthesis pathway (GFAT1; p-GFAT1, S243; NAGK) and of glutamine (GS, GLS), nucleotide (CAD), glucose (HK II), and lipid (ACC) metabolism. **(D)** Quantification of the targets assayed in **(C)**. Glutamine:fructose 6-phosphate Amidotransferase, GFAT1; Glutamine synthetase, GS; Hexokinase II, HK II; acetyl-CoA carboxylase, ACC; Glutaminase, GLS; carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, dihydroorotase, CAD; N-acetyl-Dglucosamine kinase, NAGK.

impacted organoid viability in both PDX models and had a dose-dependent effect.

mTORC1/2 Blockade Alters Multiple Metabolic Pathways in Advanced PCa Organoids *in vitro*

To determine the mTOR targets that become inhibited by our drug treatment, we performed western blot analyses on LAPC9 and BM18 organoids treated for 48 h with DMSO 0.1%, Rapalink-1 0.1–0.01 μM, rapamycin 0.1 μM, everolimus 0.1 μM, or disulfiram 0.1 μM. In both PDX models, treatment with Rapalink-1 efficiently blocked phosphorylation of the mTORC1 effectors, S6 (Ser240/244) and ULK1 (Ser757). It also abolished Akt phosphorylation (Ser473) in a dose-dependent way (**Figures 3A,B**). Although rapamycin and everolimus abolished S6 phosphorylation, they had little to no effect on ULK1 phosphorylation in both LAPC9 and BM18. They also did not

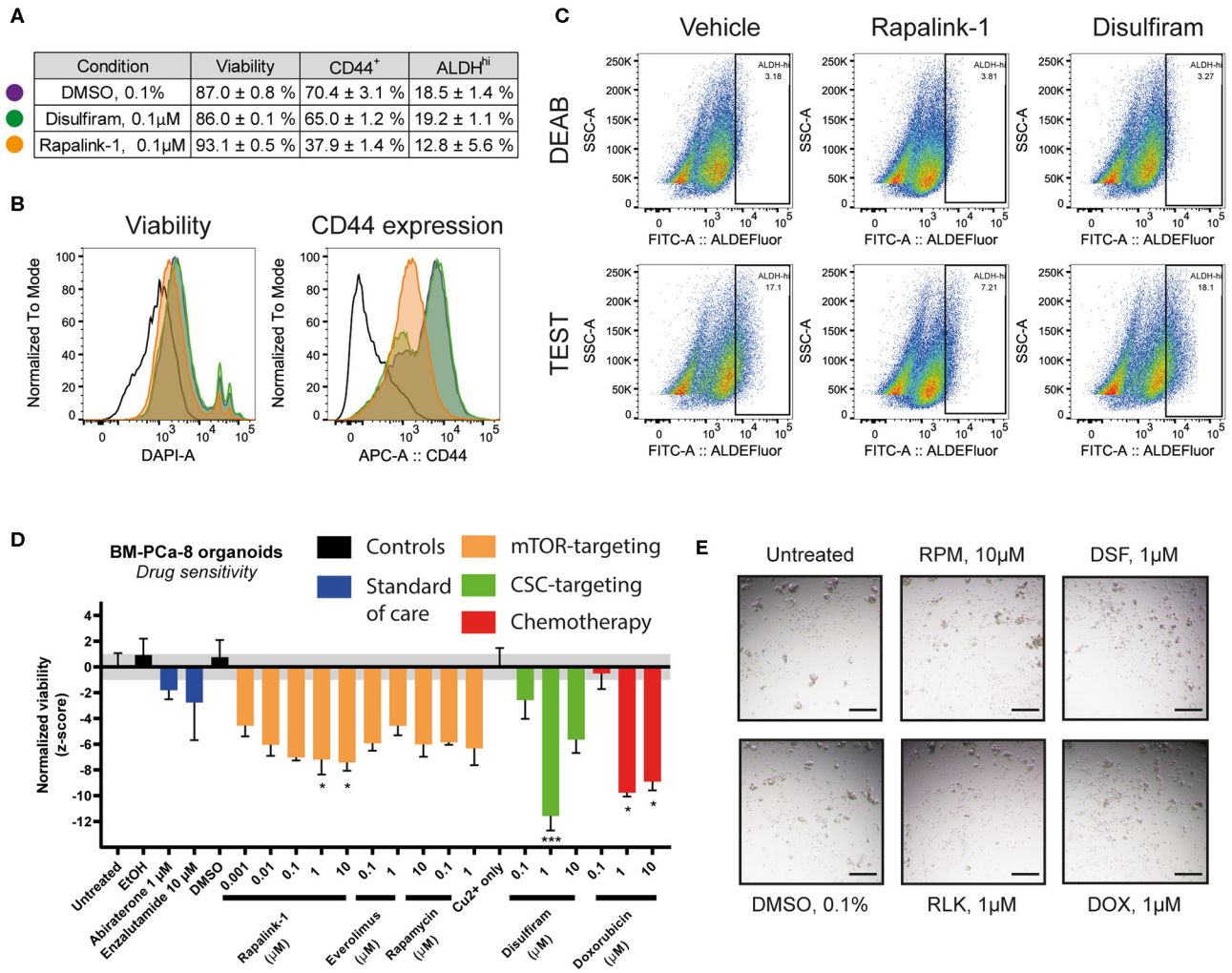
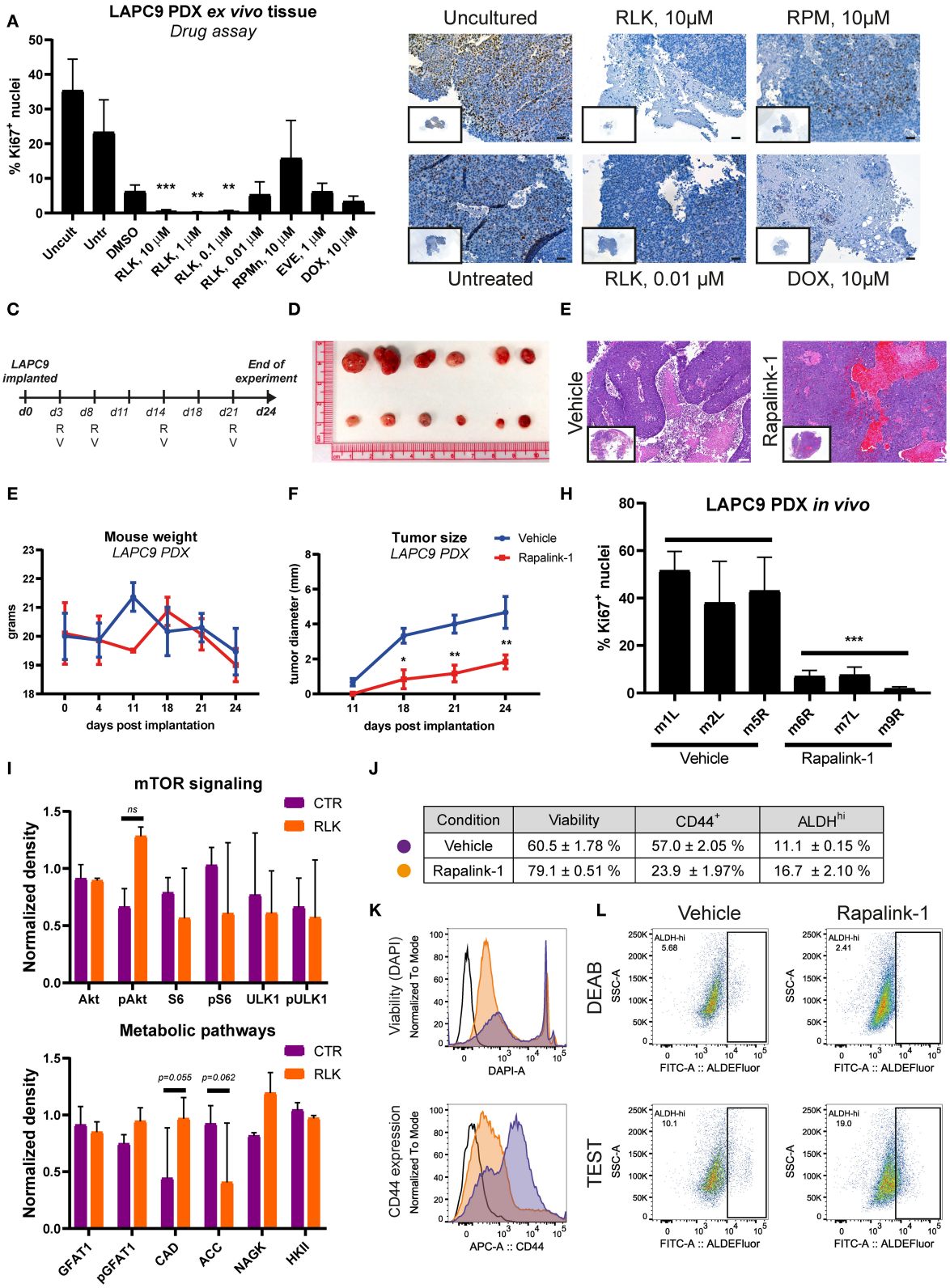


FIGURE 4 | (A) Summary table of viability values and of $CD44^{+}$ and $ALDH^{Hi}$ cells in LAPC9 organoids cultures treated with DMSO 0.1% (purple circle), disulfiram 0.1 μM (green circle) or Rapalink-1 0.1 μM (orange circle) for 48 h before analysis via flow cytometry. Data are reported as mean ± SEM, $N = 2$–5. **(B)** Representative ALDEFluor staining dot plots for $ALDH^{Hi}$ determination reported in **(A)**; open histogram, unstained; purple, DMSO; green, disulfiram; orange, Rapalink-1. Rapalink-1 reduced the $ALDH^{Hi}$ population of cells, although not significantly. **(C)** Representative viability and CD44 expression plots of data reported in **(A)**. Treatment of LAPC9 organoids with Rapalink-1 for 48 h significantly increased viability and reduced CD44 expression ($p < 0.0001$ for both analyses). Color code for the histograms is the same as reported in **(A)**. Drug screen assay on prostate bone metastasis organoids from sample BM-PCa-8. Organoids were seeded and allowed to form for 48 h before treating with the reported drugs for 48 h. **(D)** Viability values of organoids across the various tested conditions were normalized and shown. The grayed area corresponds to the expected distribution of the reference condition (untreated). Standard of care drugs (blue) were not effective treatments *in vitro* at the tested concentrations, while among the mTOR-targeting drugs (orange) the highest concentrations of rapalink-1 significantly reduced organoids viability after 48 h of treatment. Treatment with doxorubicin (1, 10 μM, red) as well as with the cancer stem cell-targeting drug disulfiram (1 μM, with 1 μM copper gluconate, green) significantly reduced PCa bone metastasis organoids viability. Data were analyzed by one-way ANOVA, treated conditions were compared to vehicle (DMSO). $N = 2$; $^{*}p < 0.05$; $^{***}p < 0.001$. **(E)** Representative images of BM-PCa-8 organoids after 48 h of treatment with the indicated drugs. Scale bars, 200 μm.



Condition	Viability	CD44+	ALDHhi
Vehicle	60.5 ± 1.78 %	57.0 ± 2.05 %	11.1 ± 0.15 %
Rapalink-1	79.1 ± 0.51 %	23.9 ± 1.97%	16.7 ± 2.10 %

FIGURE 5 | (A) Quantification of Ki67-positive nuclei stained on LAPC9 *ex vivo* tissue slices treated with the indicated compounds; a minimum of 5 fields per condition were analyzed. Data are reported as mean ± SD **(B)**. Representative images of Ki67-stained uncultured and untreated tissue, as well as of tissue treated

(Continued)

FIGURE 5 | with Rapalink-1 (RLK), rapamycin (RPM), and doxorubicin (DOX) at the reported concentrations are included, with full section enclosed in caption. Scale bar, 50 μm. **(C)** Schematic of the *in vivo* mouse experiment. R and V indicate administration of Rapalink-1 or vehicle, respectively, at the indicated day. **(D)** Picture of LAPC9 tumors explanted from Rapalink-1-treated (bottom) and vehicle-treated groups (top) at the end of experiment. Mice weight curves **(E)** and tumor size measurement **(F)** of bilateral LAPC9 PDX tumors in mice treated with vehicle (blue) or Rapalink-1 (red). For each group, $n = 3$ mice; Data are reported as mean ± SEM. **(G)** Representative HE stainings of LAPC9 tumors from vehicle-treated (top) and Rapalink-treated (bottom) mouse groups. Scale bar, 100 μm. **(H)** Quantification of Ki67-positive nuclei stained on FFPE sections of LAPC9 tissues from mice treated with Rapalink-1 or vehicle; a minimum of 9 fields per sample were analyzed. **(I)** Quantification of western blots of LAPC9 lysates from mice treated with Rapalink-1 or vehicle. Signal from assessed targets was normalized to loading control (beta actin) for each lane **(J)**. Summary table of viability values and of $CD44^+$ and $ALDH^{Hi}$ cells in LAPC9 tumors from mice treated with vehicle (purple circles) or Rapalink-1 (orange circles), analyzed by flow cytometry. Data are reported as mean ± SEM, $N = 2$–4. Representative flow cytometry plots of viability and CD44 expression are reported in **(K)**. **(L)** Representative ALDEFluor staining dot plots for $ALDH^{Hi}$ determination reported in **(J)**; open histogram, unstained; orange, Rapalink-1; purple, vehicle. LAPC9 cells from tumors of mice treated with Rapalink-1 showed a non-significant increase of $ALDH^{Hi}$ cells. A population bearing DEAB-resistant ALDH isoforms is detected in LAPC9 tumors of mice treated with vehicle (top, left panel) that is not evident in the tumors of mice treated with Rapalink-1 (top, right panel). $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

reduce Akt phosphorylation and in fact everolimus slightly enhanced Akt phosphorylation. In contrast, treatment with disulfiram had little to no effect on phosphorylation of S6 and ULK1, but reduced Akt phosphorylation in both models. Together, these findings demonstrate that Rapalink-1, but not the rapalogues or disulfiram, can effectively block both mTORC1 and mTORC2 signaling in the PDX organoids.

Since mTOR controls metabolism, we investigated how different metabolic enzymes could be affected by our drug treatment. Consistent with the robust inhibition of mTORC2 by RapaLink-1 and previous reports that mTORC2 responds to glutamine catabolites (35), we found that the metabolic enzymes that are linked to glutamine metabolism such as GFAT1, GS, GLS, and CAD were effectively diminished by RapaLink-1 (**Figures 3C,D**). Furthermore, ACC, a metabolic enzyme involved in lipid metabolism, which is also controlled by both mTORC1 and mTORC2 (36–38), was also reduced by Rapalink-1 but not by rapamycin or everolimus. On the other hand, NAGK, which is involved in the salvage hexosamine biosynthesis and HK II, which is involved in glucose metabolism were not significantly affected by any of the drug treatments. Thus, inhibiting both the mTOR complexes using Rapalink-1 can more effectively block the expression of metabolic enzymes involved in glutamine and lipid metabolism.

Combined Inhibition of mTORC1/2 Decreases Stem Cell Markers in a CRPC PDX Model and Reduces PCa Bone Metastasis PDO Viability

To further investigate the effects of the combined mTORC1/2 inhibition on the cancer stem cell (CSC) subpopulation, we treated LAPC9 organoids for 48 h with sublethal doses of Rapalink-1 (0.1 μM), comparing the effect to treatment with disulfiram (0.1 μM, with copper gluconate 1 μM) or DMSO (0.1%). The treated organoids were then analyzed with flow cytometry for viability, ALDEFluor staining and CD44 expression. A table summarizing the results is reported in **Figure 4A**.

We found that compared to DMSO, treatment with disulfiram had no impact on the assessed markers, whereas treatment with Rapalink-1 significantly reduced the CD44-positive cell fraction (from 70.4 to 37.9%, $p < 0.0001$) and increased viability (from 87.0 to 93.1%, $p < 0.0001$, **Figure 4B**). It also decreased the fraction of $ALDH^{Hi}$ cells, although with a higher variability compared to the other markers (from 18.5 to 12.8%, *ns*, **Figure 4C**). Data from the BM18 PDX were also generated and despite a trend in reduced CD44-positive cell fraction and $ALDH^{Hi}$ cells, comparing the effects of Rapalink-1 and disulfiram to DMSO yielded no significant differences (**Supplementary Figure 1**)

We functionally tested the effect of Rapalink-1 on CSC by performing a drug assay on PDO from a PCa bone metastasis sample (BM-PCa-8, **Figure 4D**). We found that treating the organoids with abiraterone or enzalutamide, two standard of care drugs normally used for the treatment of advanced castration-resistant PCa (CRPC), had no significant effect on their viability, supporting a castration-resistant profile for this sample. Doxorubicin, as well as the CSC-targeting drug disulfiram, were both effective to reduce BM-PCa organoids viability. For this latter drug, multiple cytotoxic mechanisms of action were proposed, both dependent and independent on copper that was supplemented in the disulfiram-treated wells (18). Representative pictures of the BM-PCa-8 PDO after 48 h treatment with the reported drugs are shown in **Figure 4E**.

Treatment of LAPC9 *in vivo* With Rapalink-1 Delays Tumor Growth

Before evaluating the effects of Rapalink-1 *in vivo*, we investigated its effects on a near-patient *ex vivo* tissue slices assay on the PDX LAPC9. We compared the effects of Rapalink-1 to those of rapamycin, everolimus and doxorubicin, selected as a positive control. The effect of the drugs on the proliferation marker Ki67 was measured on FFPE sections of the treated *ex vivo* tissue. Treatment with Rapalink-1 at the three highest concentrations tested (10–0.1 μM) significantly reduced LAPC9 proliferation *ex vivo*, in line with the effective inhibition of mTOR signaling and expression of several metabolic enzymes (**Figure 5A**). Representative images of the *ex vivo* LAPC9 tissues treated with the reported drugs are shown in **Figure 5B**, an image of the whole section is enclosed. Representative output images of ImageJ macro quantification after processing are reported in **Supplementary Figure 2**.

We then assessed the effect of Rapalink-1 (1.5 mg/Kg/5–7 days) *in vivo* on LAPC9 PDX model, comparing the treatment to vehicle only, a schematic of treatment schedule is reported

(**Figure 5C**). At the end of the experiment, mice treated with Rapalink-1 had significantly smaller tumors compared to mice treated with vehicle only (**Figures 5D,F**). Mice treated with Rapalink-1 did not show signs of acute toxicity throughout the experiment and had a weight curve comparable to that of vehicle-treated animals (**Figure 5E**). Basing on hematoxylin and eosin (HE) staining, LAPC9 tissues collected from the Rapalink-treated mouse group showed a lower fraction of necrotic tissue compared to stainings from the vehicle group (**Figure 5G**). Analysis of tumors from mice treated with Rapalink-1 showed a significantly lower proliferative activity, as evidenced by Ki67 staining on FFPE tumor sections (**Figure 5H**, **Supplementary Figure 3**). Protein analysis of matched tumor lysates showed diminished phosphorylation of S6 and ULK1, indicating inhibition of mTORC1. Interestingly, Akt phosphorylation was enhanced in the Rapalink-treated group, indicating that mTORC2 was active at this time point (**Figure 5I**, **Supplementary Figure 4**). Among the metabolic enzymes that we examined, there was an increase of nitrogen metabolizing enzymes CAD and NAGK and a decrease of ACC1, controlling lipid biosynthesis. In order to assess the effect of Rapalink-1 treatment on the CSC subpopulation of LAPC9, tumors from Rapalink-1 and vehicle-treated animals were digested and stained for CD44 expression and with ALDEFluor assay, whereas DAPI was used to measure cell viability within the analyzed samples. Two samples per condition were independently processed and acquired, a table with the results is reported together with representative plots of viability measurement and of CD44 expression (**Figure 5J**). Tumor cells from mice treated with Rapalink-1 had on average a significantly higher viability compared to tumors from vehicle-treated animals (79.1 $\pm$ 0.51% vs. 60.5 $\pm$ 1.78% alive cells, respectively, $p < 0.0001$). However, the $CD44^+$ compartment in the former samples was markedly and significantly lower (23.9 $\pm$ 1.97% vs. 57.0 $\pm$ 2.05%, respectively, $p < 0.0001$), indicating a depletion of $CD44^+$ cells in the LAPC9 tumors of mice treated with Rapalink-1 (**Figure 5K**). Unexpectedly, the ALDEFluor assay indicated an enrichment, although not significant, of $ALDH^{hi}$ cells in the Rapalink-treated tumors (16.7 $\pm$ 2.10%) compared to the vehicle-treated tumors (11.1 $\pm$ 0.15%). Of note, the ALDEFluor assay reveals that treatment of mice with Rapalink-1 induced a metabolic alteration in LAPC9 cancer cells. This was highlighted by the presence of a DEAB-resistant, ALDEFluor-reactive subpopulation of cells clearly detectable in the DEAB-treated samples of mice receiving vehicle (**Figure 5L**, top left panel). A DEAB-resistant population was not detectable in LAPC9 cells of mice treated with Rapalink-1 (**Figure 5L**, top right panel).

DISCUSSION

Despite the intense research on the mechanisms of bone metastasis formation, a consensus molecular classification of bone metastasis is still missing. Metastatic bone lesions can be histologically identified as lytic, blastic or mixed if the effect on the bone tissue is mainly erosive, sclerotic or a co-occurrence of both processes, respectively (1). In this work however, this histological classification did not match the unsupervised cluster analysis nor the PCA at the transcriptomic level, in contrast to the findings of a recent study by Ihle et al. (36). In their study, the authors compared lytic and blastic PCa bone metastasis by GSE analysis, finding the enrichment of different pathways in the lytic vs. blastic lesions. The differences found between the present study and that from Ihle *et al.* may be ascribed to the different sources used in the two settings (FFPE vs. snap-frozen tissue). We demonstrated the cytotoxic effectiveness of Rapalink-1, in comparison to doxorubicin, disulfiram and standard-of-care drugs, on PCa established PDX and near-patient bone metastasis-derived organoids. Standard-of-care drugs abiraterone and enzalutamide could not elicit a significant response in any of the tested conditions. As both the established PDX and the bone metastasis organoids are derived from advanced, bone-metastatic prostate cancer, this result might reflect convergent resistance mechanisms to AR inhibition possibly evolved during tumor progression. This is particularly significative for BM18 organoids, as this model is sensitive to AR inhibition *in vivo* (8, 35). The organoids *in vitro* culture system may enrich for a more AR independent subpopulation, as in the *in vivo* castrated state. As expected however, all organoids responded to the chemotherapeutic drug doxorubicin targeting DNA replication.

While organoids from both PDX were significantly inhibited by disulfiram concentrations above 1 μM, in bone metastasis organoids the significant cytotoxic effect shown at 1 μM was not replicated at the higher concentration of 10 μM. This could be explained by the chemistry of disulfiram, that forms cytotoxic equimolar complexes with Cu^{2+}. At 10 μM concentration the amount of uncomplexed disulfiram might have reduced the cytotoxic effect of Cu^{2+}-complexed disulfiram. More significantly, the sublethal dose of 0.1 μM disulfiram tested *in vitro* on LAPC9 and BM18 organoids failed to significantly modulate CSC features like CD44 expression or $ALDH^{Hi}$ cell fraction. Overall, these results suggest multiple mechanisms of action of disulfiram, that could be linked to concentration and bioavailability.

Activating mutations in different components of the PI3K/Akt pathway occur in 49% of mCRPC, including mutations of *PTEN* (>40% of cases), and are solidly implicated in PCa progression (37, 38). The modulation of the PI3K/Akt/mTOR pathway during PCa progression also correlates with alterations in the AR pathway and the cross-talk of these two pathways is currently the focus of active research (27, 39, 40). Increased mTOR signaling is associated with lymph node progression and increased lymphangiogenesis in advanced prostate cancer, supporting a link between mTOR activation and metastatic spread of PCa (41). We confirmed the activation of the PI3K/Akt/mTOR pathway in our group of bone metastatic PCa samples. Noteworthy, in all models tested, including the PCa bone metastasis, we found increased sensitivity to Rapalink-1 compared to rapamycin and the rapalog everolimus. Several clinical trials utilizing rapalogs either as monotherapy or combination therapy revealed clinical inefficacy in the treatment of prostate cancer (28) as well as other types of cancer (19). Rapalogs only inhibit a subset of mTORC1 targets and thus have cytostatic rather than cytotoxic effects. Hence, mTOR inhibitors that block mTOR kinase activity

have been engineered to more fully inhibit mTOR functions. Since the mTOR kinase domain displays homology to PI3K catalytic domain, dual PI3K/mTOR inhibitors have also been developed for better targeted therapy. However, despite the potent effect of mTOR and PI3K/mTOR inhibitors in cellular models, they have less durable effect *in vivo*, thus necessitating increased dose leading to toxicity (29). Rapalink-1 was developed to combine the durable effect of rapalogs (owing to binding with FKBP12) and robust inhibition of both mTORC1 and mTORC2 (31). The effect of Rapalink-1 in abolishing phosphorylation of mTORC1 (S6, ULK1) and mTORC2 (Akt) effectors was dose-proportionate and coincided with the robust overall decrease in cell viability of the PDX organoids. This was accompanied by significant reduction of expression of metabolic enzymes that have been linked to mTOR signaling, in particular glutamine-requiring pathways and lipid metabolism (19, 20). It is notable that we also found enrichment of genes relating to lipid metabolism and Myc. The latter is involved in increased glutamine metabolism in a number of cancers (42). Hence, it is possible that growth of the bone metastatic PCa organoids is highly dependent on mTOR-mediated glutamine- and/or lipid metabolism, making them particularly susceptible to combined mTORC1/2 inhibition.

The analysis of LAPC9 tumor lysates from mice treated with Rapalink-1 indicates residual mTORC2 activity as well as fewer metabolic alterations compared to Rapalink-1-treated organoids. Despite this divergence, treatment of mice with Rapalink-1 every 6 days was sufficient to significantly reduce tumor growth, as assessed by both tumor size and Ki67 staining on lysates-matched tumor sections. Of note, the analyzed tumor lysates from the Rapalink-1-treated mice group showed that the effects on mTOR activation and lipid metabolism regulation were heterogeneous (**Supplementary Figure 4**). This could be explained by differences in Rapalink-1 bioavailability among the particular mice, owing to factors like varying tumor size, structure or vascularization, as well as by the onset of compensatory mechanisms in tumors from treated mice. Moreover, both LAPC9 PDX and organoids treated with Rapalink-1 showed a significant decrease of $CD44^+$ cells, indicating not only a direct cytotoxic effect of the treatment, but also the alteration of PCa subpopulation homeostasis. In line with this observation, *in vivo* Rapalink-1 treatment altered the expression of aldehyde dehydrogenases (ALDH) in the surviving cells, as evidenced by the ALDEFluor assay. In the assay, the large-spectrum ALDH inhibitor DEAB is provided together with a fluorogenic substrate detecting multiple ALDH isoforms. However, the DEAB does not inhibit all isoforms of ALDH (17), an effect that was evident in the reported results. Recently, Vaddi et al. published a study linking functional CSC traits of multiple PCa cell lines to an enriched PI3K/Akt/mTOR pathway both at the RNA and at the protein levels (43). Of note, pharmacological inhibition of the PI3K/Akt pathway was associated with a reduction of the CSC population *in vitro*, in line with previous reports from Dubrovska et al. (44, 45). The effect of Rapalink-1 on different LAPC9 subpopulations could also explain the small but significant increase of viability detected both *in vitro* at sublethal doses of Rapalink-1 and *in vivo*. In both cases, the dose of Rapalink-1 used could have had a direct cytotoxic effect on the more mTOR-addicted subpopulations, selecting or inducing a subset of CD44-low, metabolically slow cells.

Compared to breast cancer, PCa bone metastasis were also enriched for pathways involved in oxidative phosphorylation and lipid metabolism (fatty acid metabolism, peroxisome, adipogenesis, cholesterol homeostasis), a finding in line with an increase in lipid metabolism in more advanced PCa stages (46) and supporting the clinical relevance of targeting this metabolic branch to prevent the development of androgen-resistance (47). An altered lipid metabolism has been linked to CSC for multiple cancer types (48). Given the substrate preferences of the different ALDH isoforms (49), it would be interesting to determine if upregulation of the mTOR pathway induced metabolic rewiring in PCa cells, or metabolic diversification of subpopulations within the tumor. Further experiments are required to support this hypothesis.

In conclusion, we provided a molecular analysis of a group of breast and prostate cancer bone metastasis and showed the translational applicability of an organoid-based drug screen on patient-derived bone metastatic tissue. We demonstrated the effectiveness of the dual mTORC1-2 inhibitor Rapalink-1 in reducing PCa tumor growth, an effect that was associated with the depletion of $CD44^+$ cells in a PDX model of advanced, bone metastatic PCa.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: European Genome Archive (https://ega-archive.org/ - Project ID: EGAS00001004431).

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements.

AUTHOR CONTRIBUTIONS

FL collected and analyzed most of the data, contributed in designing the experimental aspects. SK, MDM, and FL carried over the animal experiments and generated the data for PDX transcriptomic. IK generated the histological data. MDF analyzed the transcriptomic data. NP and EJ generated, analyzed, and interpreted proteomic data. PK and LB provided the clinical data and specimens. FL, MK, and EJ wrote the manuscript and designed figures. RP provided critical revision of the article. MK and GT supervised the project. MK conceived and designed the study. All authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.01012/full#supplementary-material

REFERENCES

1. Macedo F, Ladeira K, Pinho F, Saraiva N, Bonito N, Pinto L, et al. Bone metastases: an overview. *Oncol Rev*. (2017) 11:321. doi: 10.4081/oncol.2017.321
2. Croucher PI, McDonald MM, Martin TJ. Bone metastasis: the importance of the neighbourhood. *Nat Rev Cancer*. (2016) 16:373–86. doi: 10.1038/nrc.2016.44
3. Weilbaecher KN, Guise TA, McCauley LK. Cancer to bone: a fatal attraction. *Nat Rev Cancer*. (2011) 11:411–25. doi: 10.1038/nrc3055
4. Mehra R, Kumar-Sinha C, Shankar S, Lonigro RJ, Jing X, Philips NE, et al. Characterization of bone metastases from rapid autopsies of prostate cancer patients. *Clin Cancer Res*. (2011) 17:3924–32. doi: 10.1158/1078-0432.CCR-10-3120
5. Van Allen EM, Foye A, Wagle N, Kim W, Carter SL, McKenna A, et al. Successful whole-exome sequencing from a prostate cancer bone metastasis biopsy. *Prostate Cancer Prostatic Dis*. (2014) 17:23–7. doi: 10.1038/pcan.2013.37
6. Roubaud G, Liaw BC, Oh WK, Mulholland DJ. Strategies to avoid treatment-induced lineage crisis in advanced prostate cancer. *Nat Rev Clin Oncol*. (2017) 14:269–83. doi: 10.1038/nrclinonc.2016.181
7. Brennen WN, Isaacs JT. The what, when, and why of human prostate cancer xenografts. *Prostate*. (2018) 78:646–54. doi: 10.1002/pros.23510
8. McCulloch DR, Opeskin K, Thompson EW, Williams ED. BM18: A novel androgen-dependent human prostate cancer xenograft model derived from a bone metastasis. *Prostate*. (2005) 65:35–43. doi: 10.1002/pros.20255
9. Craft N, Chhor C, Tran C, Belldegrun A, DeKernion J, Witte ON, et al. Evidence for clonal outgrowth of androgen-independent prostate cancer cells from androgen-dependent tumors through a two-step process. *Cancer Res*. (1999) 59:5030–6.
10. Pece S, Tosoni D, Confalonieri S, Mazzarol G, Vecchi M, Ronzoni S, et al. Biological and molecular heterogeneity of breast cancers correlates with their cancer stem cell content. *Cell*. (2010) 140:62–73. doi: 10.1016/j.cell.2009.12.007
11. Marcucci F, Rumio C, Lefoulon F. Anti-cancer stem-like cell compounds in clinical development - an overview and critical appraisal. *Front Oncol*. (2016) 6:115. doi: 10.3389/fonc.2016.00115
12. Jagust P, De Luxán-Delgado B, Parejo-Alonso B, Sancho P. Metabolism-based therapeutic strategies targeting cancer stem cells. *Front Pharmacol*. (2019) 10:203. doi: 10.3389/fphar.2019.00203
13. Sharpe B, Beresford M, Bowen R, Mitchard J, Chalmers AD. Searching for prostate cancer stem cells: markers and methods. *Stem Cell Rev Rep*. (2013) 9:721–30. doi: 10.1007/s12015-013-9453-4
14. Qin J, Liu X, Laffin B, Chen X, Choy G, Jeter CR, et al. The PSA-/lo prostate cancer cell population harbors self-renewing long-term tumor-propagating cells that resist castration. *Cell Stem Cell*. (2012) 10:556–69. doi: 10.1016/j.stem.2012.03.009
15. Magnen C Le, Bubendorf L, Rentsch CA, Mengus C, Gsponer J, Zellweger T, et al. Characterization and clinical relevance of ALDH bright populations in prostate cancer. *Clin Cancer Res*. (2013) 19:5361–71. doi: 10.1158/1078-0432.CCR-12-2857
16. Yan J, De Melo J, Cutz JC, Aziz T, Tang D. Aldehyde dehydrogenase 3A1 associates with prostate tumorigenesis. *Br J Cancer*. (2014) 110:2593–603. doi: 10.1038/bjc.2014.201
17. Zhou L, Sheng D, Wang D, Ma W, Deng Q, Deng L, et al. Identification of cancer-type specific expression patterns for active aldehyde dehydrogenase (ALDH) isoforms in ALDEFLUOR assay. *Cell Biol Toxicol*. (2019) 35:161–77. doi: 10.1007/s10565-018-9444-y
18. Triscott J, Rose Pambid M, Dunn SE. Concise review: bullseye: targeting cancer stem cells to improve the treatment of gliomas by repurposing disulfiram. *Stem Cells*. (2015) 33:1042–6. doi: 10.1002/stem.1956
19. Magaway C, Kim E, Jacinto E. Targeting mTOR and metabolism in Cancer: lessons and innovations. *Cells*. (2019) 8:1584. doi: 10.3390/cells8121584
20. Mossmann D, Park S, Hall MN. mTOR signalling and cellular metabolism are mutual determinants in cancer. *Nat Rev Cancer*. (2018) 18:744–57. doi: 10.1038/s41568-018-0074-8
21. Janku F, Yap TA, Meric-Bernstam F. Targeting the PI3K pathway in cancer: are we making headway? *Nat Rev Clin Oncol*. (2018) 15:273–91. doi: 10.1038/nrclinonc.2018.28
22. Liu P, Cheng H, Roberts TM, Zhao JJ. Targeting the phosphoinositide 3-kinase pathway in cancer. *Nat Rev Drug Discov*. (2009) 8:627–44. doi: 10.1038/nrd2926
23. Stambolic V, Tsao M-S, Macpherson D, Suzuki A, Chapman WB, Mak TW. High incidence of breast and endometrial neoplasia resembling human cowden syndrome in pten+/– Mice. *Cancer Res*. (2000) 60:3605–11. Available online at: https://cancerres.aacrjournals.org/content/60/13/3605
24. Carver BS, Chapinski C, Wongvipat J, Hieronymus H, Chen YY, Chandarlapaty S, et al. Reciprocal feedback regulation of PI3K and androgen receptor signaling in pten-deficient prostate cancer. *Cancer Cell*. (2011) 19:575–86. doi: 10.1016/j.ccr.2011.04.008
25. Mulholland DJ, Tran LM, Li Y, Cai H, Morim A, Wang S, et al. Cell autonomous role of PTEN in regulating castration-resistant prostate cancer growth. *Cancer Cell*. (2011) 19:792–804. doi: 10.1016/j.ccr.2011.05.006
26. Wang X, Julio MKD, Economides KD, Walker D, Yu H, Halili MVV, et al. A luminal epithelial stem cell that is a cell of origin for prostate cancer. *Nature*. (2009) 461:495–500. doi: 10.1038/nature08361
27. Audet-Walsh É, Dufour CR, Yee T, Zouanat FZ, Yan M, Kalloghlian G, et al. Nuclear mTOR acts as a transcriptional integrator of the androgen signaling pathway in prostate cancer. *Genes Dev*. (2017) 31:1228–42. doi: 10.1101/gad.299958.117
28. Statz CM, Patterson SE, Mockus SM. mTOR inhibitors in castration-resistant prostate cancer: a systematic review. *Targeted Oncol*. (2017) 12:47–59. doi: 10.1007/s11523-016-0453-6
29. Wei XX, Hsieh AC, Kim W, Friedlander T, Lin AM, Louttit M, et al. A phase I study of abiraterone acetate combined with BEZ235, a dual PI3K/mTOR inhibitor, in metastatic castration resistant prostate cancer. *Oncologist*. (2017) 22:503-e43. doi: 10.1634/theoncologist.2016-0432
30. Graham L, Banda K, Torres A, Carver BS, Chen Y, Pisano K, et al. A phase II study of the dual mTOR inhibitor MLN0128 in patients with metastatic castration resistant prostate cancer. *Invest New Drugs*. (2018) 36:458–67. doi: 10.1007/s10637-018-0578-9
31. Rodrik-Outmezguine VS, Okaniwa M, Yao Z, Novotny CJ, McWhirter C, Banaji A, et al. Overcoming mTOR resistance mutations with a new-generation mTOR inhibitor. *Nature*. (2016) 534:272–6. doi: 10.1038/nature17963
32. Beltran H, Eng K, Mosquera JM, Sigaras A, Romanel A, Rennert H, et al. Whole-exome sequencing of metastatic cancer and biomarkers of treatment response. *JAMA Oncol*. (2015) 1:466–74. doi: 10.1001/jamaoncol.2015.1313
33. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*. (2013) 29:15–21. doi: 10.1093/bioinformatics/bts635
34. Nikolayeva O, Robinson MD. edgeR for differential RNA-seq and ChIP-seq analysis: an application to stem cell biology. *Methods Mol Biol*. (2014) 1150:45–79. doi: 10.1007/978-1-4939-0512-6_3
35. Karkampouna S, La Manna F, De Filippo MR, Kiener M, De Menna M, Zoni E, et al. Patient-derived xenografts and organoids model therapy response in prostate cancer. *bioRxiv [Pre-print]*. (2020). doi: 10.1101/2020.03.17.994350

36. Ihle CL, Provera MD, Straign DM, Smith EE, Edgerton SM, Van Bokhoven A, et al. Distinct tumor microenvironments of lytic and blastic bone metastases in prostate cancer patients. *J Immunother Cancer.* (2019) 7:293. doi: 10.1186/s40425-019-0753-3
37. Taylor BS, Schultz N, Hieronymus H, Gopalan A, Xiao Y, Carver BS, et al. Integrative genomic profiling of human prostate cancer. *Cancer Cell.* (2010) 18:11–22. doi: 10.1016/j.ccr.2010.05.026
38. Robinson D, Van Allen EM, Wu YM, Schultz N, Lonigro RJ, Mosquera JM, et al. Integrative clinical genomics of advanced prostate cancer. *Cell.* (2015) 161:1215–28. doi: 10.1016/j.cell.2015.05.001
39. Bitting RL, Armstrong AJ. Targeting the PI3K/Akt/mTOR pathway in castration-resistant prostate cancer. *Endocr Relat Cancer.* (2013) 20:R83–99. doi: 10.1530/ERC-12-0394
40. Blattner M, Liu D, Robinson BD, Huang D, Poliakov A, Gao D, et al. SPOP mutation drives prostate tumorigenesis *in vivo* through coordinate regulation of PI3K/mTOR and AR signaling. *Cancer Cell.* (2017) 31:436–51. doi: 10.1016/j.ccell.2017.02.004
41. Lilis I, Giopanou I, Papadaki H, Gyftopoulos K. The expression of p-mTOR and COUP-TFII correlates with increased lymphangiogenesis and lymph node metastasis in prostate adenocarcinoma. *Urol Oncol Semin Orig Investig.* (2018) 36:311.e27–35. doi: 10.1016/j.urolonc.2018.02.007
42. Deberardinis RJ, Cheng T. Q's next: the diverse functions of glutamine in metabolism, cell biology and cancer. *Oncogene.* (2010) 29:313–24. doi: 10.1038/onc.2009.358
43. Vaddi PK, Stamnes MA, Cao H, Chen S. Elimination of SOX2/OCT4-associated prostate cancer stem cells blocks tumor development and enhances therapeutic response. *Cancers.* (2019) 11:1331. doi: 10.3390/cancers11091331
44. Dubrovska A, Kim S, Salamone RJ, Walker JR, Maira SM, García-Echeverría C, et al. The role of PTEN/Akt/PI3K signaling in the maintenance and viability of prostate cancer stem-like cell populations. *Proc Natl Acad Sci USA.* (2009) 106:268–73. doi: 10.1073/pnas.0810956106
45. Dubrovska A, Elliott J, Salamone RJ, Kim S, Aimone LJ, Walker JR, et al. Combination therapy targeting both tumor-initiating and differentiated cell populations in prostate carcinoma. *Clin Cancer Res.* (2010) 16:5692–702. doi: 10.1158/1078-0432.CCR-10-1601
46. Zadra G, Photopoulos C, Loda M. The fat side of prostate cancer. *Biochim Biophys Acta.* (2013) 1831:1518–32. doi: 10.1016/j.bbalip.2013.03.010
47. Zadra G, Ribeiro CF, Chetta P, Ho Y, Cacciatore S, Gao X, et al. Inhibition of *de novo* lipogenesis targets androgen receptor signaling in castration-resistant prostate cancer. *Proc Natl Acad Sci USA.* (2019) 116:631–40. doi: 10.1073/pnas.1808834116
48. Visweswaran M, Arfuso F, Warrier S, Dharmarajan A. Concise review: aberrant lipid metabolism as an emerging therapeutic strategy to target cancer stem cells. *Stem Cells.* (2019) 38:6–14. doi: 10.1002/stem.3101
49. Koppaka V, Thompson DC, Chen Y, Ellermann M, Nicolaou KC, Juvonen RO, et al. Aldehyde dehydrogenase inhibitors: a comprehensive review of the pharmacology, mechanism of action, substrate specificity, and clinical application. *Pharmacol Rev.* (2012) 64:520–39. doi: 10.1124/pr.111.005538

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REVIEW
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Endoplasmic Reticulum Stress in Bone Metastases

Longyong Xu[1,2,3], *Weijie Zhang*[1,2,3], *Xiang H.-F. Zhang*[1,2,3] *and Xi Chen*[1,2,3]*

[1] Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, United States, [2] Lester and Sue Smith Breast Center, Baylor College of Medicine, Houston, TX, United States, [3] Dan L. Duncan Cancer Center, Baylor College of Medicine, Houston, TX, United States

Metastases—the spreading of cancer cells from primary tumors to distant organs, including bone—is often incurable and is the major cause of morbidity in cancer patients. Understanding how cancer cells acquire the ability to colonize to bone and become overt metastases is critical to identify new therapeutic targets and develop new therapies against bone metastases. Recent reports indicate that the endoplasmic reticulum (ER) stress and, as its consequence, the unfolded protein response (UPR) is activated during metastatic dissemination. However, their roles in this process remain largely unknown. In this review, we discuss the recent progress on evaluating the tumorigenic, immunoregulatory and metastatic effects of ER stress and the UPR on bone metastases. We explore new opportunities to translate this knowledge into potential therapeutic strategies for patients with bone metastases.

Keywords: bone metastases, seed and soil, metastatic niche, ER stress, unfolded protein response, immunotherapy

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Harvard Medical School, United States

***Correspondence:**
Xi Chen
Xi.Chen@bcm.edu

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INTRODUCTION

Bone is a frequent site of cancer metastases, and skeletal metastasis is much more common than the primary bone cancers (1). Metastatic spread of primary tumor cells to bone tissues comprises the following multiple-step cascade: (1) local invasion at the primary site; (2) intravasation; (3) survival in circulation; (4) arrest at distant organ sites; (5) extravasation to enter the parenchymal tissues of distant organs; (6) survival in the new microenvironment; and (7) proliferation to form macroscopic, clinically detectable secondary tumors, which is the step that eventually leads to morbidity (**Figure 1**) (2–4). Considerable research efforts have demonstrated that both intrinsic traits of cancer cells (the seeds) and the unique bone microenvironmental factors (the soil) contribute to the development of bone metastases (1, 3, 5–7). These efforts have led to approved treatment on bone metastases, exemplified by the introduction of bisphosphonates and denosumab (8–10). Meanwhile, several clinical trials are on-going based on the knowledge from these efforts. Further studies aim to understanding the molecular basis for each step of bone metastasis will be instrumental to manage the bone metastasis.

Tumor cells endure intrinsic (oncogenic) and extrinsic environmental stresses during metastatic dissemination (11). These stresses can either increase the protein synthesis, overwhelming the protein folding capacity of the endoplasmic reticulum (ER) or directly disrupt ER protein folding. This leads to the accumulation of unfolded and misfolded proteins (known as ER stress) (12, 13). An adaptive mechanism, termed the unfolded protein response (UPR), is consequently initiated by transmembrane sensors on the ER upon detection of ER stress to restore ER homeostasis (14). Multiple functions of the UPR in the development of primary tumors have been extensively studied, and targeting the UPR has been shown to be an effective therapeutic strategy in multiple cancers

(15–28). However, its role in metastases remain far less documented. In this review, we will discuss the mechanisms of the UPR in tumor progression and its potential implications in bone metastases.

BONE METASTASES

The relative incidence, median survival, and effect on bone homeostasis (osteolytic, osteoblastic, or mixed) of bone metastases vary greatly among different cancer types (**Table 1**) (1, 29, 30). Bone metastases are associated with multiple skeletal complications, including bone pain, impaired mobility, pathologic fractures, nerve compression, bone marrow aplasia, and hypercalcemia (31). The clinical detection of metastases may be a late event of disease progression; although, the dissemination to bone may occur early. Disseminated tumor cells (DTCs) can be detected in the bone marrow of patients and in mouse models even without the invasive diseases (32–38).

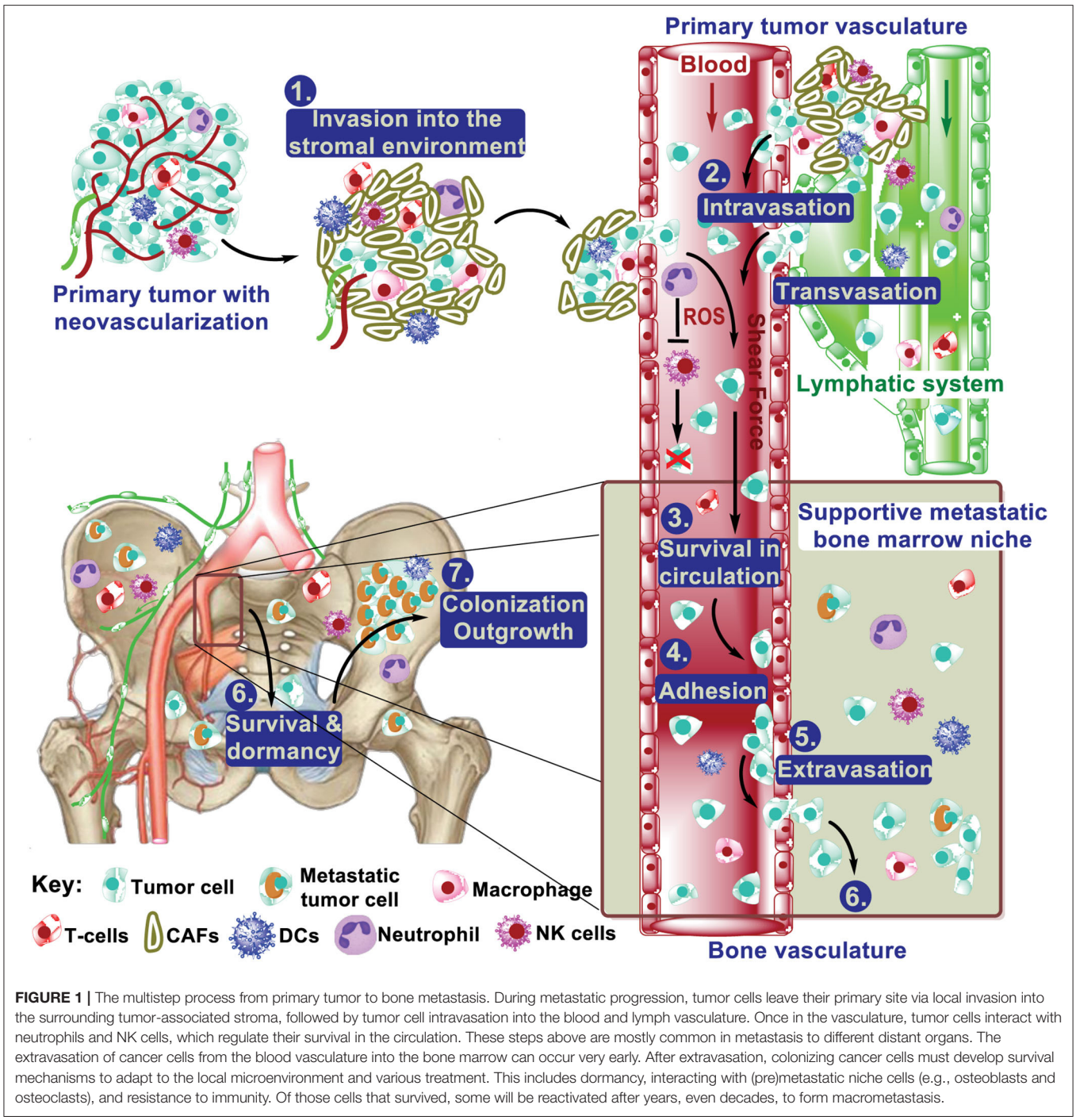

FIGURE 1 | The multistep process from primary tumor to bone metastasis. During metastatic progression, tumor cells leave their primary site via local invasion into the surrounding tumor-associated stroma, followed by tumor cell intravasation into the blood and lymph vasculature. Once in the vasculature, tumor cells interact with neutrophils and NK cells, which regulate their survival in the circulation. These steps above are mostly common in metastasis to different distant organs. The extravasation of cancer cells from the blood vasculature into the bone marrow can occur very early. After extravasation, colonizing cancer cells must develop survival mechanisms to adapt to the local microenvironment and various treatment. This includes dormancy, interacting with (pre)metastatic niche cells (e.g., osteoblasts and osteoclasts), and resistance to immunity. Of those cells that survived, some will be reactivated after years, even decades, to form macrometastasis.

TABLE 1 | Incidence of bone metastases in cancer (1).

Primary cancer type	Relative incidence	Median survival (months)	Five-year survival rate	Impact to bone homeostasis
Breast	65–75%	19–25	20%	Mixed
Prostate	65–75%	12–53	25%	Osteoblastic
Lung	30–40%	6	< 5%	Osteolytic (NSCLC) Osteoblastic (SCLC)
Thyroid	40–60%	48	40%	Mixed
Bladder	40%	6–9	3%	NA
Renal	20–25%	6–12	10%	Osteolytic
Melanoma	14–45%	< 6	< 5%	Osteolytic

NSCLC, non-small-cell lung cancer; SCLC, small-cell lung cancer.

However, because many more DTCs than macrometastases are present in the bone marrow in both patients and mouse models, it remains unclear whether these early DTCs ultimately cause metastatic outgrowth or if they are simply bystanders during the metastatic process (5, 9). Nevertheless, it is clear that the presence of DTCs in bone marrow is associated with poor prognosis and predicts eventual metastases to the bone as well as other organs (39–43). The microenvironment in primary tumors contribute to the selection of secondary tumors in bone. For example, cancer-associated fibroblasts select Src-hyperactivated bone metastatic seeds in triple-negative breast cancers (TNBC) (44). Meanwhile, premetastatic niches in the bone microenvironment are actively formed by secreted factors and/or exosomes from the primary tumor prior to DTC seeding (5, 45, 46). Upon arrival at the bone, DTCs that survive the hostile environment interact with the bone resident cells, forming the metastatic niche that determines the fate of DTCs (dormant, reactivated, or drug resistant) (5, 7, 9, 47).

ER STRESS AND THE UPR

The ER is the major organelle in eukaryotic cells responsible for intracellular Ca^{2+} homeostasis, lipid biosynthesis, and the folding of membrane and secreted proteins (14, 48–52). Protein folding in the ER is precisely regulated and highly sensitive to alterations in the protein load, mutations that affect the folding process, and the ER folding environment (e.g., redox state, nutrient status, and Ca^{2+} levels) (49, 53). The accumulation of unfolded or misfolded proteins in the ER causes ER stress, which can be detected and resolved by the UPR (48, 51, 54–57). There are three major UPR signaling branches named after their transmembrane sensors: (1) inositol-requiring enzyme 1α (IRE1α, encoded by *ERN1*), (2) PKR-like ER kinase (PERK, encoded by *EIF2AK3*), and (3) activating transcription factor 6α (ATF6α, encoded by *ATF6*) (58) (**Figure 2**). All of these three sensors are activated upon the dissociation of the binding immunoglobin protein (BiP, encode by *HSPA5*) (59) or by the direct binding of unfolded proteins (60, 61) under ER stress.

IRE1α is the most evolutionarily conserved branch of the UPR (62, 63). It is a bifunctional transmembrane kinase/endoribonuclease that dimerizes, and autophosphorylates upon luminal activation and then specifically cleaves 26 nucleotides from cytoplasmic X-box binding protein 1 (*XBP1*) mRNA (*Hac1* in yeast) (54, 55, 64–66). This is the first step of a cytoplasmic splicing event that creates an active form of the transcription factor XBP1s which, among its various functions activates multiple ER quality control genes to enhance the protein folding capacity of the ER to reduce the misfolded proteins there. Meanwhile, activated IRE1α also degrades certain ER-localized cytoplasmic mRNAs in a process known as regulated IRE1-dependent decay (RIDD) to reduce the number of proteins entering the ER (67, 68). By interacting with different adaptor and modulator proteins, IER1α can also activate the JNK, ERK, p38, and NF-kB pathways (69–71).

PERK is a serine/threonine kinase, and its best characterized substrate is eIF2α (72). PERK-dependent phosphorylation of eIF2α reduces the protein load into the ER by inhibiting the 5′ cap-dependent translation, while selectively increasing the translation of ATF4. ATF4 subsequently activates multiple genes involved in the regulation of autophagy, amino acid metabolism, and antioxidant responses (73–75).

Under ER stress, ATF6 is translocated to the Golgi apparatus, where it is cleaved by site 1 protease (S1P) and S2P, releasing the cytoplasmic transcription factor fragment (76). ATF6 activates genes that are involved in protein folding in the ER. Collectively, the consequences of UPR activation—pro-survival or pro-apoptotic—depend on the duration and intensity of the stress stimuli (14, 15, 20, 51, 53, 56).

THE UPR IN PREMETASTATIC NICHE FORMATION

Survival and outgrowth of tumor cells in distant organs depend on their interaction with the microenvironment of the distal site (5–7). Several fundamental discoveries have revealed that cancer cells can remotely reprogram the microenvironments in distant organs to facilitate the later colonization, survival, and growth in a process termed prometastatic niche (PMN) formation (45, 46, 77). In the context of bone metastases, lysyl oxidase (LOX) is secreted by estrogen receptor–negative (ER^-) breast tumors and mediates PMN formation in the bone (45). Hypoxia signature is correlated with increased risk of bone metastases, particularly in ER^- breast tumors. Cox et al. found LOX is highly expressed in bone-tropic MDA-MB-231 subline 1833-BoT cells and is associated with bone tropism in ER^- breast tumors. LOX secreted by the hypoxic primary tumor leads to

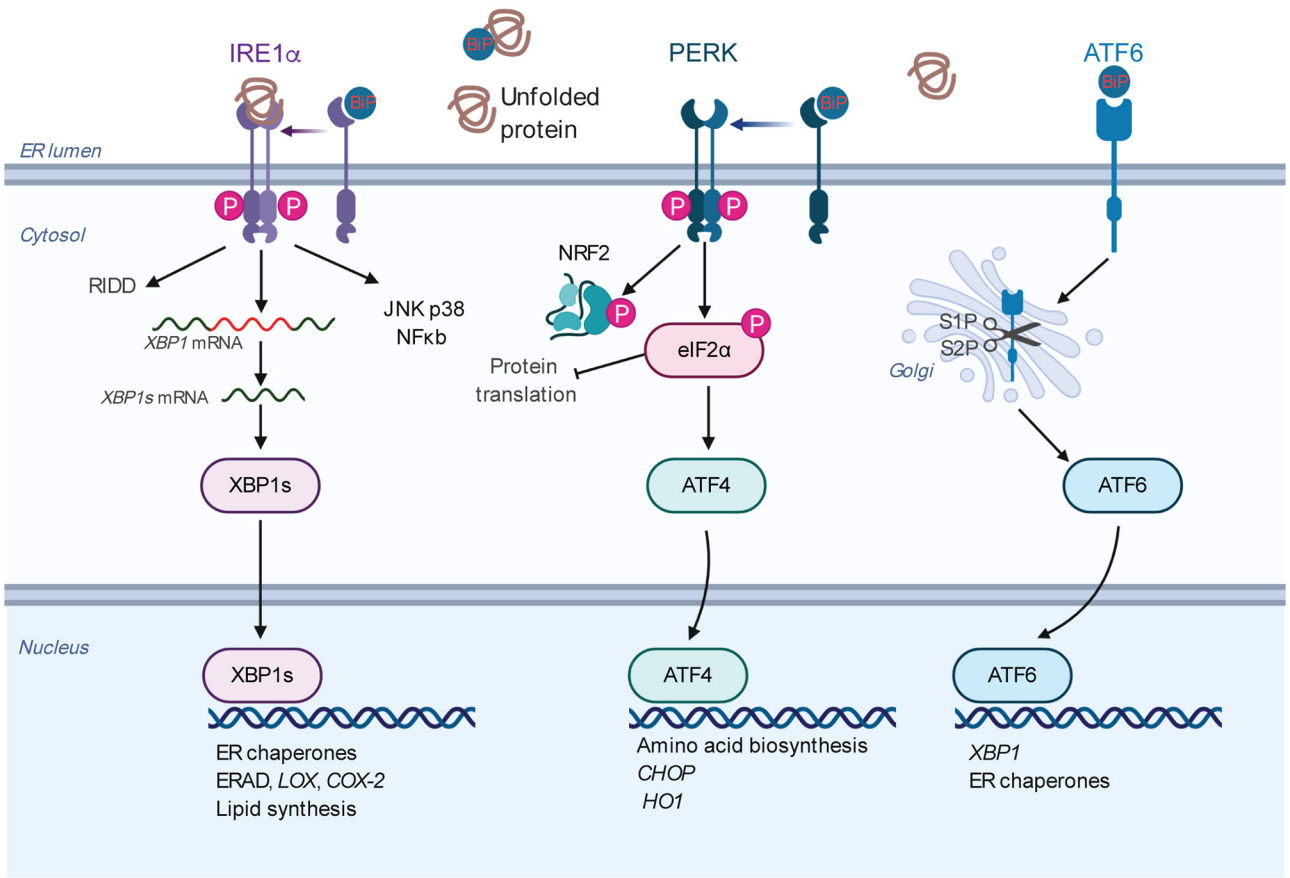

FIGURE 2 | Overview of the mammalian UPR. The three ER resident sensors (IRE1α, PERK, and ATF6) transduce information about the protein folding status of the ER to the cytosol and nucleus to restore the protein folding capacity. Under normal conditions, the sensors are inactivated by binding to the chaperone BiP. Under ER stress conditions, the sensors are activated by BiP dissociation and/or direct misfolded protein binding. Each pathway uses a different mechanism for signal transduction upon activation. IRE1α dimerizes, autophosphorylates, and triggers its RNase activity. This leads to the splicing of the XBP1 mRNA to produce an active transcription factor, spliced XBP1 (XBP1s). XBP1s induces the transcription of the genes encoding protein chaperones, ERAD, and phospholipid synthesis. The RNase activity of IRE1α also degrades certain mRNAs through RIDD. Activated IRE1α can activate the JNK, p38, ERK, and NF-kB pathways, thus playing an XBP1-independent role to modulate diverse cellular responses. Upon activation, PERK phosphorylates eIF2α, leading to global translational attenuation while selectively mediating translation of *ATF4.* In turn, ATF4 induces the expression of genes involved in amino acid metabolism, proapoptotic factor DDIT3/CHOP, and antioxidant responses (HO1). PERK also phosphorylates and stabilizes NRF2, a transcription factor involved in redox metabolism. ATF6 is transported to the Golgi apparatus under ER stress, where it is processed by S1P and S2P, releasing its cytosolic domain fragment as a transcription factor. ATF6 activates genes encoding protein chaperones, ERAD components, *and XBP1.* Abbreviations: ATF, activating transcription factor; BiP, binding immunoglobulin protein; *DDIT3,* DNA damage inducible transcript 3; eIF2α, eukaryotic translation initiation factor 2 subunit 1; ER, endoplasmic reticulum; ERAD, ER-associated protein degradation; HO1, heme oxygenase 1; IRE1α, inositol-requiring enzyme 1α; JNK, c-Jun N-terminal kinase; NF-kB, nuclear factor kappa light-chain enhancer of activated B cells; NRF2, NF-E2-related factor 2; PERK, PKR-like ER kinase; RIDD, regulated IRE1α dependent decay of mRNA; S1P and S2P, site 1 and site 2 proteases; UPR, unfolded protein response.

the formation of premetastatic osteolytic lesions and promotes bone metastatic burden in a 4T1-BALB/c mouse model. The expression of *LOX* is induced by hypoxia inducible factor (HIF) under hypoxic conditions (45). Meanwhile, the UPR is known to be induced by hypoxia (15). Further study demonstrated that XBP1s interacts with HIF1α and is required for the upregulation of HIF1α-mediated hypoxia response pathway genes in TNBC tumors (18). This study implies that XBP1s may directly regulate the expression of *LOX* under hypoxic conditions. Indeed, XBP1 activates *LOX* expression in lung adenocarcinoma cells to promote the cell growth (78). Thus, the increased secretion of LOX in hypoxic tumors may be due to the activation of the UPR. It is compelling to note that blocking the IRE1a-XBP1 pathway may simultaneously inhibit the growth of both the primary TNBC tumors and bone metastases. Additional work will be required to test the universality of UPR involvement in the PMN formation during bone metastasis.

ROLE OF THE UPR ON THE SURVIVAL OF CIRCULATING TUMOR CELLS

In the vasculature, circulating tumor cells (CTCs) encounter various stresses, including the loss of extracellular matrix (ECM)

detachment, oxidative stress, innate immune response, and physical shear force (3, 79, 80). Normally, cells undergo apoptosis when they lose contact with their ECM or neighboring cells. This specific type of apoptosis, termed anoikis, prevents adherent-independent cell growth, attachment to an inappropriate matrix, and thus colonization of distant organs (79, 80). Multiple studies suggest that the PERK-eIF2α branch of the UPR inhibits anoikis and is required for tumors to invade and metastasize (81–83). The PERK-eIF2α pathway was shown to activate in suspension-cultured MCF10A cells and sustains MCF10A cell survival (81). Cells that undergo epithelial-to-mesenchymal transition (EMT) are highly secretory, and the PERK axis of the UPR was found to be selectively activated (82). In addition, the inhibition of PERK compromised the ability of EMT cells to form tumorspheres and migrate in transwell assays (82). Human melanoma cells experience higher levels of oxidative stress in the circulation and distant tissues than in primary tumors (84). To manage such oxidative stress, metastasizing melanomas undergo reversible metabolic adaptations, including the synthesis of antioxidants, to survive and eventually metastasize to distant sites. A previous study (83) showed that the PERK downstream transcription factor ATF4 and NRF2, which is stabilized by PERK (85), activate the expression of major antioxidant enzyme heme oxygenase 1 (HO-1), and therefore protect the detached cells from oxidative stress.

CTCs are also vulnerable to immune attacks by innate immune cells, notably NK cells (86, 87). In contrast to NK cells, neutrophils seem to protect CTCs and favor the metastatic spreading (4, 88). The functions of the IRE1α-XBP1 branch during the CTC stage are complicated (89–92). On the one hand, XBP1s promotes NK cell proliferation and positively regulates cytolytic activity of NK cells (89, 91, 92). On the other hand, XBP1 stimulates the expression of lectin-type oxidized LDL receptor 1 (LOX-1) in human neutrophils and transforms them into immunosuppressive cells, possibly promoting CTC survival (90). Overall, the PERK pathway could promote CTC survival by inhibiting anoikis and oxidative stress. Further *in vivo* studies are necessary to evaluate the overall effect of the IRE1α-XBP1 branch on the survival of CTCs *in vivo*.

ROLE OF THE UPR ON COLONIZATION AND DORMANCY

CTCs surviving in the circulation arrive at the bone marrow vasculatures and extravasate into bone marrow parenchyma. It is still unclear whether this process is completed by passive entry due to the discontinuous endothelium of bone marrow sinusoids or if any other pathway actively involved (6, 7, 93). Compared with other organs, the bone is unique for its mineral content, enriched vasculatures, low oxygen level, high local Ca^{2+} concentration, and acidosis (94). As a result, the newly arrived tumor cells are challenged in many aspects (5, 6). Meanwhile, DTCs in bone remain dormant state in a variable period, which is critical for their survival, adaptation, escaping systemic treatments, and final outgrowth (6, 9, 94).

The hostile microenvironment (e.g., hypoxia) in the bone may disrupt ER protein folding; therefore, UPR pathways are expected to be upregulated in these DTCs. Indeed, UPR target genes are upregulated in dormant cancer cells from patients and mouse models (95–99). In the bone marrow of breast cancer patients, both GRP78/BiP and GRP94 are selectively highly expressed by bone marrow (BM) DTCs (98). Interestingly, UPR target genes are also overexpressed in cells derived from bone marrow DTCs compared with those from primary tumors (98, 100). These studies suggest that UPR upregulation is a stable trait for BM DTCs.

This trait may arise from the selection of pre-existing UPR positive subpopulation by the hostile microenvironment (and treatment, see discussion below) from the heterogenous cancer cell population, adaptation of the survived cancer cells to the microenvironment, or both. Nevertheless, these UPR genes are thought to confer a survival advantage to DTCs within the bone microenvironment because cell lines derived from BM DTCs are more resistant to glucose and oxygen deprivation *in vitro*. Studies in the head and neck cancer cell line HEp3 indicated that p38 plays a critical role in the induction and maintenance of tumor dormancy (95–97, 101). Interestingly, p38 activates all three branches of UPR in the dormant HEp3 cells, which contributes to the survival of cancer cells under glucose deprivation or chemotherapeutic treatments. Meanwhile, the PERK pathway inhibits the translation of cyclin D1/D3 and CDK4 in these cells, thereby arresting the cells in the G0-G1 phase. These studies support a causal role for the UPR in the establishment of dormancy (95–97). The upregulation of UPR genes are also found in dormant pancreatic ductal adenocarcinoma DTCs from lives of patient samples and mouse model (99). Collectively, these data indicate that UPR activation may be a common strategy utilized by cancer cells to enter dormancy and promote their survival. Further studies would be worthwhile to follow up on these impressive results and answer the following questions: (1) what triggers and/or maintains UPR signaling in dormant cancer cells in which overall protein synthesis is attenuated (101); (2) can UPR activation contribute to the dormant state of bone marrow DTCs *in vivo*, and if so, how; (3) what determines the pro-survival or pro-apoptotic effects of UPR activation in these cells; and (4) can the inhibition of the UPR promote DTCs death or sensitize them to therapies targeting proliferating cells.

ROLE OF THE UPR ON THE REACTIVATION AND OUTGROWTH OF DTCs

Our current knowledge about the reactivation process of dormant DTCs, particularly in bone, is limited (4–6, 102). The autonomous traits of tumor cells alone cannot explain the asynchronized relapse of metastases after a long latency. Alternatively, local stimulation of the microenvironments may awaken dormant tumor cells. In bone, osteoclasts are key players in the microenvironmental support of osteolytic breast cancer cell growth and bone destruction. The upregulation of vascular cell adhesion molecule 1 (VCAM-1) in tumor cells promotes the

transition from indolent micrometastasis to overt metastasis in breast cancer (103). DTCs with high VCAM-1 recruit integrin $\alpha4\beta^+$ osteoclast progenitors and induce local osteoclast activity. Therapeutically targeting the VCAM-1–integrin a4 interaction effectively inhibits the progression of bone metastasis and preserves bone structure in mouse models (103). Osteoblasts are another cell type found in the remodeling bone environment. It has been suggested that cancer cells interact with osteogenic cells through E-cadherin/N-cadherin and gap junctions and such interaction promotes early-stage bone colonization and outgrowth (104, 105). In multiple myeloma, XBP1 is required for the expression of VCAM-1, IL6, and RANKL and promotes osteolytic outgrowth (106). Given that XBP1 is one of the top transcription factors enriched in bone metastases compared with primary tumors and metastases in other organs (105), additional research is necessary to determine whether XBP1 regulates VCAM-1 in bone metastasis and promotes outgrowth.

Similarly to metastases in other organs, the immune system is absolutely critical in regulating the outgrowth of bone metastasis (94). In clinical practice, donor-derived cancer develops on rare occasions in immune-suppressed recipients who have received organs from cancer survivors (disease free for more than 10 years) or donors without diagnosable cancer at the time of transplantation (107–109). These observations suggest that the competent immune system may hold disseminated tumor cells in an asymptomatic state. Indeed, a higher ratio of $CD56^+$ $CD8^+$ T cells and memory $CD4^+$ T cells were found in DTC-present bone marrow samples than in DTC-free samples in breast cancer patients (110). In a mouse model of spontaneous bone metastasis, the restoration of interferon regulatory factor 7 (Irf7) suppresses bone metastases through interferon signaling, whereas the deficiency of T and NK cell responses accelerates breast cancer bone metastases (111). A possible interpretation of these results is that cancer cell proliferation is balanced by immune-mediated cancer cell death (4, 112). The bone marrow is occupied by diverse immune cells including neutrophils (113–115). With age, hematopoietic stem cells gradually lose their self-renewal and regeneration capacity and are biased to differentiate into myeloid lineage including monocytes (giving rise to macrophages and dendritic cells), granulocytes (giving rise to basophils, neutrophils, and eosinophils), and megakaryocytes (116–118). This leads to an aged-related decline of the immune response (referred to as immunosenescence) and chronic, sterile, low-grade inflammation (named "inflamm-aging") in older adults (119, 120). Inflammation is linked to the relapse of breast cancer (121). Sustained experimental inflammation and the accompanying formation of neutrophil extracellular traps in the lungs was found to convert dormant breast cancer cells to aggressive lung metastases in mice. Mechanistically, the neutrophil extracellular traps associated protease neutrophil elastase and matrix metalloproteinase 9 sequentially cleaves the ECM component laminin, leading to laminin remodeling. The remodeled laminin activates α3β1-FAK signaling in dormant cancer cells to induce their reactivation (122). As discussed above, XBP1 promotes neutrophils into immunosuppressive cells. In addition, the inhibition of the IRE1α RNase activity downregulates the expression and secretion of CXCL1 in multiple breast cancer cell lines (23), indicating the possibility that IRE1α promotes neutrophil recruitment by activating CXCL1. Furthermore, the IRE1a-XBP1 pathway is required for neutrophil extracellular trap formation during infection (123) Overall, the IRE1a-XBP1 pathway is known to promote neutrophil recruitment and function. Nevertheless, additional investigation is necessary to test whether neutrophils contribute to DTC reactivation in bone and whether/how the IRE1α-XBP1 pathway is involved in this process.

Dendritic cells (DCs) are responsible for the presentation of tumor antigens to T cells and initiation of the antitumor response (124). Activated T cells, especially cytotoxic CD8+ T cells and CD4+ T helper 1 cells, attack and destroy the target tumor cells (125). However, these processes are often inhibited by tumor cells via multiple strategies including at least by silencing the antigen presentation (hiding major histocompatibility complex I (MHCI) or making dendritic cells (DCs) dysfunctional), T-cell dysfunction, and the establishment of an immunosuppressive tumor microenvironment by myeloid-derived suppressor cells (MDSCs) (124–126). In contrast to the essential role of the IRE1α-XBP1 pathway in the physiology of antigen presentation cells under homeostatic conditions (127, 128), a study by the Laurie Glimcher's laboratory uncovered XBP1s as a critical driver of tumor-associated dendritic cell (tDC) dysfunction in the ovarian cancer microenvironment (129). IRE1α activation of XBP1s, stimulated by lipid peroxidation byproducts in tDCs, leads to abnormal lipid accumulation and subsequent inhibition of the antigen-presenting capacity of tDCs. Accordingly, DC-specific XBP1 inhibition restores their immunostimulatory capacity and extends survival in tumor-bearing mice. In addition, targeting the IRE1α-XBP1 pathway benefits T-cell function directly in the ovarian cancer microenvironment by increasing mitochondrial respiration activity (130) and attenuating cholesterol-induced $CD8^+$ T-cell exhaustion (131). The PERK downstream target *Chop* (encode by Ddit3) is highly expressed in tumor-associated MDSCs, and the depletion of Chop compromises the function of MDSCs and delays tumor growth (132). Therefore, inhibition of the IRE1α and PERK pathways could boost the immune response in multiple tumors.

Taken together, these recent findings suggest that ER stress is induced in tumor cells and infiltrated immune cells in the tumor microenvironment. Thus, it would be interesting to test whether the abovementioned functions of the UPR are specific to the tumor microenvironment studied or can be generalized to other cancer types and different metastatic organs including bone.

THERAPEUTIC RESISTANCE AND METASTATIC-RELATED MORBIDITY

Metastatic cancer often represents a terminal illness and is the main cause of cancer death (133). Current treatments for metastatic lesions are essentially similar to those for the corresponding primary tumors, including chemotherapy, targeted therapy, hormone therapy, radiation therapy, and immunotherapy (4, 134). Unfortunately, therapeutic resistance often occurs (4) due to many mechanisms, including tumor

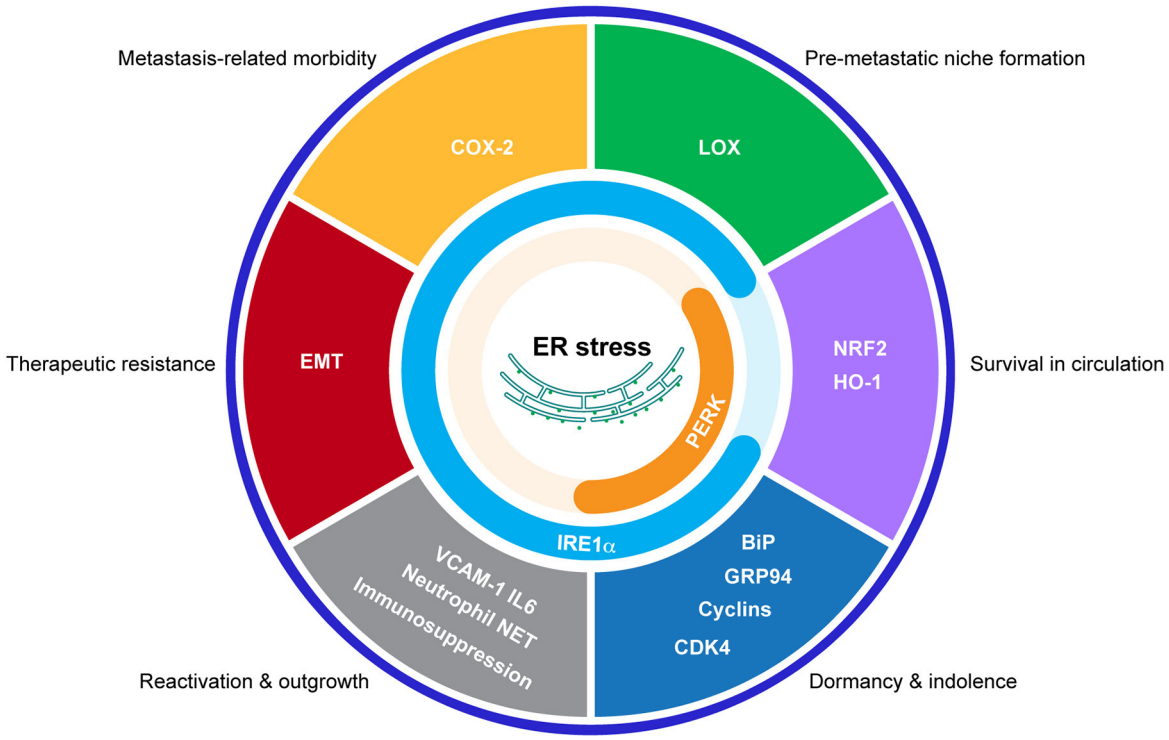

FIGURE 3 | Potential connections between the UPR and bone metastasis. Schematic representation of the proposed effects of the UPR in bone metastasis by regulating indicated processes.

dormancy, stem-like properties, EMT, and immune suppression as discussed above. In a mouse model of spontaneous lung metastases from mammary tumors, *IRE1α* expression was induced upon cyclophosphamide-mediated chemotherapy (135). This result is further supported by a study that reported that IRE1α RNase activity is induced upon paclitaxel treatment in TNBC cells (23). Importantly, the inhibition of IRE1α RNase activity increases paclitaxel-mediated tumor suppression and delays tumor relapse posttherapy (23). This is consistent with our recent finding that the inhibition of IRE1α RNase activity substantially enhances the efficacy of docetaxel-based chemotherapy in treating MYC-overexpressing primary tumors and lung metastases (26). In summary, hijacking and upregulation of the IRE1α-XBP1 pathway is one strategy tumor cells use to develop chemoresistance, yet further investigation is required on the detailed mechanisms about how this pathway is activated and how it leads to resistance.

Bone pain is one of the most frequent symptoms of bone metastases, impairing both life quality and expectancy (136). One of the extensively studied molecules that leads to bone pain is cyclooxygenase-2 (COX-2), which is the key enzyme in prostaglandin biosynthesis (136, 137). Prostaglandins bind to prostanoid receptors on sensory terminals, resulting in bone pain (136). Inhibition of COX-2 attenuates bone pain, tumor growth, and bone destruction in a mouse model (138). The two latter phenotypes can be explained by the fact that prostaglandins can also directly promote cancer cell proliferation and induce immunosuppression (137). Recently, the IRE1α-XBP1 pathway was identified as an important regulator in prostaglandin biosynthesis and pain management (139). In myeloid cells (including macrophages and monocytes), XBP1 directly activates the expression of *COX-2* and *mPGES-1*. Genetically or pharmacologically inhibition of the IRE1α-XBP1 pathway diminished pain-related behaviors in mouse models. Given the established functions of COX2/PGES-1 in pain and immunosuppression, this finding not only revealed a new therapeutic approach for attenuating pain behavior but also indicated an alternative explanation how the IRE1α-XBP1 arm promotes immunosuppression.

CONCLUSIONS AND FUTURE DIRECTIONS

In the past decade, great strides have been made in bone metastasis research to enhance our understanding of this disease in both patients and experimental models. However, some key questions still remain unanswered. What triggers DTC dormancy and reawakening? How do DTCs evade immune cell surveillance? And ultimately, can we cure bone metastases? To address these questions, both conceptual and technological advances must be made. Improved models need to be developed that faithfully mimic the natural history of bone metastases in patients. The advancement in single-cell RNA sequencing has

broadened our knowledge about the heterogeneity of cancer and bone marrow niche cells (114, 115, 140). This technique alone or together with the metastatic-niche labeling strategy (141) will shed new light on the biology of bone metastases and may identify new therapeutic targets.

The activation of the UPR has been demonstrated to endow cancer cells with tumorigenic, metastatic, and drug-resistant capacities and provide tumors with an immunosuppressive microenvironment. Given the convincing underlying mechanisms discovered and the exciting therapeutic results so far, it would be very promising to translate our current knowledge on the functions of the UPR in primary tumors to the study of bone metastases (**Figure 3**). Further studies are required to characterize the functions of the UPR in different steps of bone metastasis and in different cancer models. What are the driver events that induce/inhibit the UPR during bone metastasis? How does the UPR interplay with other signaling during this process? Can these stress responses in cancer cells be transmitted to niche cells to promote bone metastasis (142, 143)? Importantly, due to the immunosuppressive function of the UPR and the availability of multitarget drugs, it is conceivable to combine these inhibitors to various forms of cancer immunotherapy strategies to control bone metastases.

AUTHOR CONTRIBUTIONS

LX and XC: wrote the manuscript. WZ and XZ: contributed to the bone metastasis part. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Macedo F, Ladeira K, Pinho F, Saraiva N, Bonito N, Pinto L, et al. Bone metastases: an overview. *Oncol Rev*. (2017) 11:321. doi: 10.4081/oncol.2017.321
2. Valastyan S, Weinberg RA. Tumor metastasis: molecular insights and evolving paradigms. *Cell*. (2011) 147:275–92. doi: 10.1016/j.cell.2011.09.024
3. Massague J, Obenauf AC. Metastatic colonization by circulating tumour cells. *Nature*. (2016) 529:298–306. doi: 10.1038/nature17038
4. Lambert AW, Pattabiraman DR, Weinberg RA. Emerging biological principles of metastasis. *Cell*. (2017) 168:670–91. doi: 10.1016/j.cell.2016.11.037
5. Croucher PI, Mcdonald MM, Martin TJ. Bone metastasis: the importance of the neighbourhood. *Nat Rev Cancer*. (2016) 16:373–86. doi: 10.1038/nrc.2016.44
6. Esposito M, Guise T, Kang Y. The biology of bone metastasis. *Cold Spring Harb Perspect Med*. (2018) 8:a031252. doi: 10.1101/cshperspect.a031252
7. Zhang W, Bado I, Wang H, Lo HC, Zhang XH. Bone metastasis: find your niche and fit in. *Trends Cancer*. (2019) 5:95–110. doi: 10.1016/j.trecan.2018.12.004
8. Esposito M, Kang Y. Targeting tumor-stromal interactions in bone metastasis. *Pharmacol Ther*. (2014) 141:222–33. doi: 10.1016/j.pharmthera.2013.10.006
9. Goddard ET, Bozic I, Riddell SR, Ghajar CM. Dormant tumour cells, their niches and the influence of immunity. *Nat Cell Biol*. (2018) 20:1240–9. doi: 10.1038/s41556-018-0214-0
10. D'oronzo S, Coleman R, Brown J, Silvestris F. Metastatic bone disease: pathogenesis and therapeutic options: up-date on bone metastasis management. *J Bone Oncol*. (2019) 15:004–4. doi: 10.1016/j.jbo.2018.10.004
11. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. (2011) 144:646–74. doi: 10.1016/j.cell.2011.02.013
12. Wang M, Kaufman RJ. The impact of the endoplasmic reticulum protein-folding environment on cancer development. *Nat Rev Cancer*. (2014) 14:581–97. doi: 10.1038/nrc3800
13. Cubillos-Ruiz JR, Bettigole SE, Glimcher LH. Tumorigenic and immunosuppressive effects of endoplasmic reticulum stress in cancer. *Cell*. (2017) 168:692–706. doi: 10.1016/j.cell.2016.12.004
14. Walter P, Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. *Science*. (2011) 334:1081–6. doi: 10.1126/science.1209038
15. Ma Y, Hendershot LM. The role of the unfolded protein response in tumour development: friend or foe? *Nat Rev Cancer*. (2004) 4:966–77. doi: 10.1038/nrc1505
16. Romero-Ramirez L, Cao H, Nelson D, Hammond E, Lee A-H, Yoshida H, et al. XBP1 Is Essential for survival under hypoxic conditions and is required for tumor growth. *Cancer Res*. (2004) 64:5943–7. doi: 10.1158/0008-5472.CAN-04-1606
17. Hart LS, Cunningham JT, Datta T, Dey S, Tameire F, Lehman SL, et al. ER stress–mediated autophagy promotes Myc-dependent transformation and tumor growth. *J Clin Invest*. (2012) 122:4621–34. doi: 10.1172/JCI62973
18. Chen X, Iliopoulos D, Zhang Q, Tang Q, Greenblatt MB, Hatziapostolou M, et al. XBP1 promotes triple-negative breast cancer by controlling the HIF1α pathway. *Nature*. (2014) 508:103–7. doi: 10.1038/nature13119
19. Tang C-HA, Ranatunga S, Kriss CL, Cubitt CL, Tao J, Pinilla-Ibarz JA, et al. Inhibition of ER stress–associated IRE-1/XBP-1 pathway reduces leukemic cell survival. *J Clin Invest*. (2014) 124:2585–98. doi: 10.1172/JCI73448
20. Urra H, Dufey E, Avril T, Chevet E, Hetz C. Endoplasmic reticulum stress and the hallmarks of cancer. *Trends Cancer*. (2016) 2:252–62. doi: 10.1016/j.trecan.2016.03.007
21. Ojha R, Amaravadi RK. Targeting the unfolded protein response in cancer. *Pharmacol Res*. (2017) 120:258–66. doi: 10.1016/j.phrs.2017.04.003
22. Rufo N, Garg AD, Agostinis P. The unfolded protein response in immunogenic cell death and cancer immunotherapy. *Trends Cancer*. (2017) 3:643–58. doi: 10.1016/j.trecan.2017.07.002
23. Logue SE, Mcgrath EP, Cleary P, Greene S, Mnich K, Almanza A, et al. Inhibition of IRE1 RNase activity modulates the tumor cell secretome and enhances response to chemotherapy. *Nat Commun*. (2018) 9:3267. doi: 10.1038/s41467-018-05763-8
24. Nguyen HG, Conn CS, Kye Y, Xue L, Forester CM, Cowan JE, et al. Development of a stress response therapy targeting aggressive prostate cancer. *Sci Transl Med*. (2018) 10:eaar2036. doi: 10.1126/scitranslmed.aar2036
25. Xie H, Tang C-HA, Song JH, Mancuso A, Del Valle JR, Cao J, et al. IRE1α RNase–dependent lipid homeostasis promotes survival in Myc-transformed cancers. *J Clin Invest*. (2018) 128:1300–16. doi: 10.1172/JCI95864
26. Zhao N, Cao J, Xu L, Tang Q, Dobrolecki LE, Lv X, et al. Pharmacological targeting of MYC-regulated IRE1/XBP1 pathway suppresses MYC-driven breast cancer. *J Clin Invest*. (2018) 128:1283–99. doi: 10.1172/JCI95873
27. Song M, Cubillos-Ruiz JR. Endoplasmic reticulum stress responses in intratumoral immune cells: implications for cancer immunotherapy. *Trends Immunol*. (2019) 40:128–41. doi: 10.1016/j.it.2018.12.001

28. Tameire F, Verginadis II, Leli NM, Polte C, Conn CS, Ojha R, et al. ATF4 couples MYC-dependent translational activity to bioenergetic demands during tumour progression. *Nat Cell Biol*. (2019) 21:889–99. doi: 10.1038/s41556-019-0347-9
29. Arvelo F, Sojo F, Cotte C. Cancer and the metastatic substrate. *Ecancermedicalscience*. (2016) 10:701. doi: 10.3332/ecancer.2016.701
30. Svensson E, Christiansen CF, Ulrichsen SP, Rørth MR, Sørensen HT. Survival after bone metastasis by primary cancer type: a Danish population-based cohort study. *BMJ Open*. (2017) 7:e016022. doi: 10.1136/bmjopen-2017-016022
31. Weidle UH, Birzele F, Kollmorgen G, Rüger R. Molecular mechanisms of bone metastasis. *Cancer Genomics Proteomics*. (2016) 13:1–12. doi: 10.21873/cgp.20004
32. Melchior SW, Corey E, Ellis WJ, Ross AA, Layton TJ, Oswin MM, et al. Early tumor cell dissemination in patients with clinically localized carcinoma of the prostate. *Clin Cancer Res*. (1997) 3:249–56.
33. Husemann Y, Geigl JB, Schubert F, Musiani P, Meyer M, Burghart E, et al. Systemic spread is an early step in breast cancer. *Cancer Cell*. (2008) 13:58–68. doi: 10.1016/j.ccr.2007.12.003
34. Morgan TM, Lange PH, Porter MP, Lin DW, Ellis WJ, Gallaher IS, et al. Disseminated tumor cells in prostate cancer patients after radical prostatectomy and without evidence of disease predicts biochemical recurrence. *Clin Cancer Res*. (2009) 15:677–83. doi: 10.1158/1078-0432.CCR-08-1754
35. Sanger N, Effenberger KE, Riethdorf S, Van Haasteren V, Gauwerky J, Wiegratz I, et al. Disseminated tumor cells in the bone marrow of patients with ductal carcinoma in situ. *Int J Cancer*. (2011) 129:2522–6. doi: 10.1002/ijc.25895
36. Gruber IV, Hartkopf AD, Hahn M, Taran FA, Staebler A, Wallwiener D, et al. Relationship between hematogenous tumor cell dissemination and cellular immunity in DCIS patients. *Anticancer Res*. (2016) 36:2345–51.
37. Harper KL, Sosa MS, Entenberg D, Hosseini H, Cheung JF, Nobre R, et al. Mechanism of early dissemination and metastasis in Her2(+) mammary cancer. *Nature*. (2016) 540:588–92. doi: 10.1038/nature20609
38. Hosseini H, Obradovic MMS, Hoffmann M, Harper KL, Sosa MS, Werner-Klein M, et al. Early dissemination seeds metastasis in breast cancer. *Nature*. (2016) 540:552–8. doi: 10.1038/nature20785
39. Weckermann D, Müller P, Wawroschek F, Harzmann R, Riethmüller G, Schlimok G. Disseminated cytokeratin positive tumor cells in the bone marrow of patients with prostate cancer: detection and prognostic value. *J Urol*. (2001) 166:699–704. doi: 10.1016/S0022-5347(05)66046-6
40. Braun S, Vogl FD, Naume B, Janni W, Osborne MP, Coombes RC, et al. A pooled analysis of bone marrow micrometastasis in breast cancer. *N Eng J Med*. (2005) 353:793–802. doi: 10.1056/NEJMoa050434
41. Janni W, Vogl FD, Wiedswang G, Synnestvedt M, Fehm T, Jückstock J, et al. Persistence of disseminated tumor cells in the bone marrow of breast cancer patients predicts increased risk for relapse—a european pooled analysis. *Clin Cancer Res*. (2011) 17:2967–76. doi: 10.1158/1078-0432.CCR-10-2515
42. Gundem G, Van Loo P, Kremeyer B, Alexandrov LB, Tubio JMC, Papaemmanuil E, et al. The evolutionary history of lethal metastatic prostate cancer. *Nature*. (2015) 520:353–7. doi: 10.1038/nature14347
43. Hoadley KA, Siegel MB, Kanchi KL, Miller CA, Ding L, Zhao W, et al. Tumor evolution in two patients with basal-like breast cancer: a retrospective genomics study of multiple metastases. *PLoS Med*. (2016) 13:e1002174. doi: 10.1371/journal.pmed.1002174
44. Zhang XH, Jin X, Malladi S, Zou Y, Wen YH, Brogi E, et al. Selection of bone metastasis seeds by mesenchymal signals in the primary tumor stroma. *Cell*. (2013) 154:1060–73. doi: 10.1016/j.cell.2013.07.036
45. Cox TR, Rumney RMH, Schoof EM, Perryman L, Høye AM, Agrawal A, et al. The hypoxic cancer secretome induces pre-metastatic bone lesions through lysyl oxidase. *Nature*. (2015) 522:106–10. doi: 10.1038/nature14492
46. Peinado H, Zhang H, Matei IR, Costa-Silva B, Hoshino A, Rodrigues G, et al. Pre-metastatic niches: organ-specific homes for metastases. *Nat Rev Cancer*. (2017) 17:302–17. doi: 10.1038/nrc.2017.6
47. Celià-Terrassa T, Kang Y. Metastatic niche functions and therapeutic opportunities. *Nat Cell Biol*. (2018) 20:868–77. doi: 10.1038/s41556-018-0145-9
48. Malhotra JD, Kaufman RJ. The endoplasmic reticulum and the unfolded protein response. *Semin Cell Dev Biol*. (2007) 18:716–31. doi: 10.1016/j.semcdb.2007.09.003
49. Braakman I, Hebert DN. Protein folding in the endoplasmic reticulum. *Cold Spring Harb Perspect Biol*. (2013) 5:a013201. doi: 10.1101/cshperspect.a013201
50. Jan CH, Williams CC, Weissman JS. Principles of ER cotranslational translocation revealed by proximity-specific ribosome profiling. *Science*. (2014) 346:1257521. doi: 10.1126/science.1257521
51. Bettigole SE, Glimcher LH. Endoplasmic reticulum stress in immunity. *Annu Rev Immunol*. (2015) 33:107–38. doi: 10.1146/annurev-immunol-032414-112116
52. Schwarz DS, Blower MD. The endoplasmic reticulum: structure, function and response to cellular signaling. *Cell Mol Life Sci*. (2016) 73:79–94. doi: 10.1007/s00018-015-2052-6
53. Braakman I, Bulleid NJ. Protein Folding and Modification in the Mammalian Endoplasmic Reticulum. *Annu Rev Biochem*. (2011) 80:71–99. doi: 10.1146/annurev-biochem-062209-093836
54. Cox JS, Walter P. A novel mechanism for regulating activity of a transcription factor that controls the unfolded protein response. *Cell*. (1996) 87:391–404. doi: 10.1016/S0092-8674(00)81360-4
55. Mori K, Kawahara T, Yoshida H, Yanagi H, Yura T. Signalling from endoplasmic reticulum to nucleus: transcription factor with a basic-leucine zipper motif is required for the unfolded protein-response pathway. *Genes Cells*. (1996) 1:803–17. doi: 10.1046/j.1365-2443.1996.d01-274.x
56. Hetz C. The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nat Rev Mol Cell Biol*. (2012) 13:89–102. doi: 10.1038/nrm3270
57. Almanza A, Carlesso A, Chintha C, Creedican S, Doultsinos D, Leuzzi B, et al. Endoplasmic reticulum stress signalling – from basic mechanisms to clinical applications. *FEBS J*. (2019) 286:241–78. doi: 10.1111/febs.14608
58. Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol Cell Biol*. (2007) 8:519–29. doi: 10.1038/nrm2199
59. Bertolotti A, Zhang Y, Hendershot LM, Harding HP, Ron D. Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nat Cell Biol*. (2000) 2:326–32. doi: 10.1038/35014014
60. Gardner BM, Walter P. Unfolded proteins are Ire1-activating ligands that directly induce the unfolded protein response. *Science*. (2011) 333:1891–4. doi: 10.1126/science.1209126
61. Karagöz GE, Acosta-Alvear D, Nguyen HT, Lee CP, Chu F, Walter P. An unfolded protein-induced conformational switch activates mammalian IRE1. *Elife*. (2017) 6:e30700. doi: 10.7554/eLife.30700.034
62. Cox JS, Shamu CE, Walter P. Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase. *Cell*. (1993) 73:1197–206. doi: 10.1016/0092-8674(93)90648-A
63. Morl K, Ma W, Gething M-J, Sambrook J. A transmembrane protein with a cdc2+CDC28-related kinase activity is required for signaling from the ER to the nucleus. *Cell*. (1993) 74:743–56. doi: 10.1016/0092-8674(93)90521-Q
64. Shamu CE, Walter P. Oligomerization and phosphorylation of the Ire1p kinase during intracellular signaling from the endoplasmic reticulum to the nucleus. *EMBO J*. (1996) 15:3028–39. doi: 10.1002/j.1460-2075.1996.tb00666.x
65. Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell*. (2001) 107:881–91. doi: 10.1016/S0092-8674(01)00611-0
66. Calfon M, Zeng H, Urano F, Till JH, Hubbard SR, Harding HP, et al. IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature*. (2002) 415:92–6. doi: 10.1038/415092a
67. Hollien J, Weissman JS. Decay of endoplasmic reticulum-localized mRNAs during the unfolded protein response. *Science*. (2006) 313:104–7. doi: 10.1126/science.1129631
68. Hollien J, Lin JH, Li H, Stevens N, Walter P, Weissman JS. Regulated Ire1-dependent decay of messenger RNAs in mammalian cells. *J Cell Biol*. (2009) 186:323–31. doi: 10.1083/jcb.200903014
69. Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP, et al. Coupling of stress in the ER to activation of JNK protein

kinases by transmembrane protein kinase IRE1. *Science*. (2000) 287:664–6. doi: 10.1126/science.287.5453.664
70. Nguyên DT, Kebache S, Fazel A, Wong HN, Jenna S, Emadali A, et al. Nck-dependent activation of extracellular signal-regulated kinase-1 and regulation of cell survival during endoplasmic reticulum stress. *Mol Biol Cell*. (2004) 15:4248–60. doi: 10.1091/mbc.e03-11-0851
71. Hu P, Han Z, Couvillon AD, Kaufman RJ, Exton JH. Autocrine tumor necrosis factor alpha links endoplasmic reticulum stress to the membrane death receptor pathway through IRE1α-mediated NF-κB activation and down-regulation of TRAF2 expression. *Mol Cell Biol*. (2006) 26:3071–84. doi: 10.1128/MCB.26.8.3071-3084.2006
72. Harding HP, Zhang Y, Ron D. Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. *Nature*. (1999) 397:271–4. doi: 10.1038/16729
73. Lu PD, Harding HP, Ron D. Translation reinitiation at alternative open reading frames regulates gene expression in an integrated stress response. *J Cell Biol*. (2004) 167:27–33. doi: 10.1083/jcb.200408003
74. Vattem KM, Wek RC. Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. *Proc Natl Acad Sci USA*. (2004) 101:11269–74. doi: 10.1073/pnas.0400541101
75. Liu Z, Lv Y, Zhao N, Guan G, Wang J. Protein kinase R-like ER kinase and its role in endoplasmic reticulum stress-decided cell fate. *Cell Death Dis*. (2015) 6:e1822. doi: 10.1038/cddis.2015.183
76. Haze K, Yoshida H, Yanagi H, Yura T, Mori K. Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. *Mol Biol Cell*. (1999) 10:3787–99. doi: 10.1091/mbc.10.11.3787
77. Hoshino A, Costa-Silva B, Shen T-L, Rodrigues G, Hashimoto A, Tesic Mark M, et al. Tumour exosome integrins determine organotropic metastasis. *Nature*. (2015) 527:329–35. doi: 10.1038/nature15756
78. Yang Y, Cheng BJ, Jian H, Chen ZW, Zhao Y, Yu YF, et al. XBP1-LOX Axis is critical in ER stress-induced growth of lung adenocarcinoma in 3D culture. *Am J Transl Res*. (2017) 9:700–7.
79. Simpson CD, Anyiwe K, Schimmer AD. Anoikis resistance and tumor metastasis. *Cancer Lett*. (2008) 272:177–85. doi: 10.1016/j.canlet.2008.05.029
80. Paoli P, Giannoni E, Chiarugi P. Anoikis molecular pathways and its role in cancer progression. *Biochim Biophys Acta*. (2013) 1833:3481–98. doi: 10.1016/j.bbamcr.2013.06.026
81. Avivar-Valderas A, Salas E, Bobrovnikova-Marjon E, Diehl JA, Nagi C, Debnath J, et al. PERK integrates autophagy and oxidative stress responses to promote survival during extracellular matrix detachment. *Mol Cell Biol*. (2011) 31:3616–29. doi: 10.1128/MCB.05164-11
82. Feng YX, Sokol ES, Del Vecchio CA, Sanduja S, Claessen JHL, Proia TA, et al. Epithelial-to-mesenchymal transition activates PERK–eIF2α and sensitizes cells to endoplasmic reticulum stress. *Cancer Discov*. (2014) 4:702–15. doi: 10.1158/2159-8290.CD-13-0945
83. Dey S, Sayers CM, Verginadis II, Lehman SL, Cheng Y, Cerniglia GJ, et al. ATF4-dependent induction of heme oxygenase 1 prevents anoikis and promotes metastasis. *J Clin Invest*. (2015) 125:2592–608. doi: 10.1172/JCI78031
84. Piskounova E, Agathocleous M, Murphy MM, Hu Z, Huddlestun SE, Zhao Z, et al. Oxidative stress inhibits distant metastasis by human melanoma cells. *Nature*. (2015) 527:186–91. doi: 10.1038/nature15726
85. Cullinan SB, Zhang D, Hannink M, Arvisais E, Kaufman RJ, Diehl JA. Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. *Mol Cell Biol*. (2003) 23:7198–209. doi: 10.1128/MCB.23.20.7198-7209.2003
86. Hanna N, Fidler IJ. Role of natural killer cells in the destruction of circulating tumor Emboli23. *J Natl Cancer Inst*. (1980) 65:801–9. doi: 10.1093/jnci/65.4.801
87. López-Soto A, Gonzalez S, Smyth MJ, Galluzzi L. Control of metastasis by NK cells. *Cancer Cell*. (2017) 32:135–54. doi: 10.1016/j.ccell.2017.06.009
88. Szczerba BM, Castro-Giner F, Vetter M, Krol I, Gkountela S, Landin J, et al. Neutrophils escort circulating tumour cells to enable cell cycle progression. *Nature*. (2019) 566:553–7. doi: 10.1038/s41586-019-0915-y
89. Mujaj S, Gandhi M, Vari F, Nourse J. Modulation of the unfolded protein response via XBP1 splicing: a novel mechanism that regulates natural killer cell effector function. (172.10). *J Immunol*. (2012) 188:172.110.
90. Condamine T, Dominguez GA, Youn JI, Kossenkov AV, Mony S, Alicea-Torres K, et al. Lectin-type oxidized LDL receptor-1 distinguishes population of human polymorphonuclear myeloid-derived suppressor cells in cancer patients. *Sci Immunol*. (2016) 1:aaf8943. doi: 10.1126/sciimmunol.aaf8943
91. Dong H, Adams NM, Xu Y, Cao J, Allan DSJ, Carlyle JR, et al. The IRE1 endoplasmic reticulum stress sensor activates natural killer cell immunity in part by regulating c-Myc. *Nat Immunol*. (2019) 20:865–78. doi: 10.1038/s41590-019-0388-z
92. Wang Y, Zhang Y, Yi P, Dong W, Nalin AP, Zhang J, et al. The IL-15–AKT–XBP1s signaling pathway contributes to effector functions and survival in human NK cells. *Nat Immunol*. (2019) 20:10–7. doi: 10.1038/s41590-018-0265-1
93. Acar M, Kocherlakota KS, Murphy MM, Peyer JG, Oguro H, Inra CN, et al. Deep imaging of bone marrow shows non-dividing stem cells are mainly perisinusoidal. *Nature*. (2015) 526:126–30. doi: 10.1038/nature15250
94. Johnson RW, Suva LJ. Hallmarks of bone metastasis. *Calcif Tissue Int*. (2018) 102:141–51. doi: 10.1007/s00223-017-0362-4
95. Ranganathan AC, Zhang L, Adam AP, Aguirre-Ghiso JA. Functional coupling of p38-induced up-regulation of BiP and activation of RNA-dependent protein kinase–like endoplasmic reticulum kinase to drug resistance of dormant carcinoma cells. *Cancer Res*. (2006) 66:1702–11. doi: 10.1158/0008-5472.CAN-05-3092
96. Ranganathan AC, Ojha S, Kourtidis A, Conklin DS, Aguirre-Ghiso JA. Dual function of pancreatic endoplasmic reticulum kinase in tumor cell growth arrest and survival. *Cancer Res*. (2008) 68:3260–8. doi: 10.1158/0008-5472.CAN-07-6215
97. Schewe DM, Aguirre-Ghiso JA. ATF6α-Rheb-mTOR signaling promotes survival of dormant tumor cells in vivo. *Proc Natl Acad Sci USA*. (2008) 105:10519–24. doi: 10.1073/pnas.0800939105
98. Bartkowiak K, Effenberger KE, Harder S, Andreas A, Buck F, Peter-Katalinic J, et al. Discovery of a novel unfolded protein response phenotype of cancer stem/progenitor cells from the bone marrow of breast cancer patients. *J Proteome Res*. (2010) 9:3158–68. doi: 10.1021/pr100039d
99. Pommier A, Anaparthy N, Memos N, Kelley ZL, Gouronnec A, Yan R, et al. Unresolved endoplasmic reticulum stress engenders immune-resistant, latent pancreatic cancer metastases. *Science*. (2018) 360:eaao4908. doi: 10.1126/science.aao4908
100. Bartkowiak K, Kwiatkowski M, Buck F, Gorges TM, Nilse L, Assmann V, et al. Disseminated tumor cells persist in the bone marrow of breast cancer patients through sustained activation of the unfolded protein response. *Cancer Res*. (2015) 75:5367–77. doi: 10.1158/0008-5472.CAN-14-3728
101. Ranganathan AC, Adam AP, Zhang L, Aguirre-Ghiso JA. Tumor cell dormancy induced by p38SAPK and ER-stress signaling: an adaptive advantage for metastatic cells? *Cancer Biol Ther*. (2006) 5:729–35. doi: 10.4161/cbt.5.7.2968
102. Giancotti F. Mechanisms governing metastatic dormancy and reactivation. *Cell*. (2013) 155:750–64. doi: 10.1016/j.cell.2013.10.029
103. Lu X, Mu E, Wei Y, Riethdorf S, Yang Q, Yuan M, et al. VCAM-1 promotes osteolytic expansion of indolent bone micrometastasis of breast cancer by engaging a4b1-positive osteoclast progenitors. *Cancer Cell*. (2011) 20:701–14. doi: 10.1016/j.ccr.2011.11.002
104. Wang H, Yu C, Gao X, Welte T, Muscarella AM, Tian L, et al. The osteogenic niche promotes early-stage bone colonization of disseminated breast cancer cells. *Cancer Cell*. (2015) 27:193–210. doi: 10.1016/j.ccell.2014.11.017
105. Wang H, Tian L, Liu J, Goldstein A, Bado I, Zhang W, et al. The osteogenic niche is a calcium reservoir of bone micrometastases and confers unexpected therapeutic vulnerability. *Cancer Cell*. (2018) 34:823–39 e827. doi: 10.1016/j.ccell.2018.10.002
106. Xu G, Liu K, Anderson J, Patrene K, Lentzsch S, Roodman GD, et al. Expression of XBP1s in bone marrow stromal cells is critical for myeloma cell growth and osteoclast formation. *Blood*. (2012) 119:4205–14. doi: 10.1182/blood-2011-05-353300
107. Buell JF, Beebe TM, Trofe J, Gross TG, Alloway RR, Hanaway MJ, et al. Donor transmitted malignancies. *Ann Transplant*. (2004) 9:53–6.
108. Strauss DC, Thomas JM. Transmission of donor melanoma by organ transplantation. *Lancet Oncol*. (2010) 11:790–6. doi: 10.1016/S1470-2045(10)70024-3

109. Xiao D, Craig JC, Chapman JR, Dominguez-Gil B, Tong A, Wong G. Donor cancer transmission in kidney transplantation: a systematic review. *Am J Transplant*. (2013) 13:2645–52. doi: 10.1111/ajt.12430
110. Feuerer M, Rocha M, Bai L, Umansky V, Solomayer EF, Bastert G, et al. Enrichment of memory T cells and other profound immunological changes in the bone marrow from untreated breast cancer patients. *Int J Cancer*. (2001) 92:96–105. doi: 10.1002/1097-0215(200102)9999:9999<::AID-IJC1152>3.0.CO;2-Q
111. Bidwell BN, Slaney CY, Withana NP, Forster S, Cao Y, Loi S, et al. Silencing of Irf7 pathways in breast cancer cells promotes bone metastasis through immune escape. *Nat Med*. (2012) 18:1224–31. doi: 10.1038/nm.2830
112. Endo H, Inoue M. Dormancy in cancer. *Cancer Sci*. (2019) 110:474–80. doi: 10.1111/cas.13917
113. Zhao E, Xu H, Wang L, Kryczek I, Wu K, Hu Y, et al. Bone marrow and the control of immunity. *Cell Mol Immunol*. (2012) 9:11–9. doi: 10.1038/cmi.2011.47
114. Baryawno N, Przybylski D, Kowalczyk MS, Kfoury Y, Severe N, Gustafsson K, et al. A cellular taxonomy of the bone marrow stroma in homeostasis and leukemia. *Cell*. (2019) 177:1915–32 e1916. doi: 10.1016/j.cell.2019.04.040
115. Tikhonova AN, Dolgalev I, Hu H, Sivaraj KK, Hoxha E, Cuesta-Dominguez A, et al. The bone marrow microenvironment at single-cell resolution. *Nature*. (2019) 569:222–8. doi: 10.1038/s41586-019-1104-8
116. Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. *Cell*. (2008) 132:631–44. doi: 10.1016/j.cell.2008.01.025
117. Doulatov S, Notta F, Laurenti E, Dick JE. Hematopoiesis: a human perspective. *Cell Stem Cell*. (2012) 10:120–36. doi: 10.1016/j.stem.2012.01.006
118. Geiger H, De Haan G, Florian MC. The ageing haematopoietic stem cell compartment. *Nat Rev Immunol*. (2013) 13:376–89. doi: 10.1038/nri3433
119. Franceschi C, Bonafè M, Valensin S, Olivieri F, De Luca M, Ottaviani E, et al. Inflamm-aging: An evolutionary perspective on immunosenescence. *Ann N Y Acad Sci*. (2000) 908:244–54. doi: 10.1111/j.1749-6632.2000.tb06651.x
120. Fulop T, Larbi A, Dupuis G, Le Page A, Frost EH, Cohen AA, et al. Immunosenescence and inflamm-aging as two sides of the same coin: friends or foes? *Front Immunol*. (2018) 8:1960. doi: 10.3389/fimmu.2017.01960
121. Cole SW. Chronic inflammation and breast cancer recurrence. *J Clin Oncol*. (2009) 27:3418–9. doi: 10.1200/JCO.2009.21.9782
122. Albrengues J, Shields MA, Ng D, Park CG, Ambrico A, Poindexter ME, et al. Neutrophil extracellular traps produced during inflammation awaken dormant cancer cells in mice. *Science*. (2018) 361:eaao4227. doi: 10.1126/science.aao4227
123. Abuaita BH, Sule GJ, Schultz TL, Gao F, Knight JS, O'riordan MX. The IRE1α stress signaling axis is a key regulator of neutrophil antimicrobial effector function. *bioRxiv*. (2019) 743336. doi: 10.1101/743336
124. Wculek SK, Cueto FJ, Mujal AM, Melero I, Krummel MF, Sancho D. Dendritic cells in cancer immunology and immunotherapy. *Nat Rev Immunol*. (2020) 20:7–24. doi: 10.1038/s41577-019-0210-z
125. Speiser DE, Ho PC, Verdeil G. Regulatory circuits of T cell function in cancer. *Nat Rev Immunol*. (2016) 16:599–611. doi: 10.1038/nri.2016.80
126. Fleming V, Hu X, Weber R, Nagibin V, Groth C, Altevogt P, et al. Targeting myeloid-derived suppressor cells to bypass tumor-induced immunosuppression. *Front Immunol*. (2018) 9:398. doi: 10.3389/fimmu.2018.00398
127. Iwakoshi NN, Pypaert M, Glimcher LH. The transcription factor XBP-1 is essential for the development and survival of dendritic cells. *J Exp Med*. (2007) 204:2267–75. doi: 10.1084/jem.20070525
128. Osorio F, Tavernier SJ, Hoffmann E, Saeys Y, Martens L, Vetters J, et al. The unfolded-protein-response sensor IRE-1α regulates the function of CD8α+ dendritic cells. *Nat Immunol*. (2014) 15:248–57. doi: 10.1038/ni.2808
129. Cubillos-Ruiz JR, Silberman, PC, Rutkowski, MR, Chopra S, Perales-Puchalt A, Song M, et al. ER stress sensor XBP1 controls anti-tumor immunity by disrupting dendritic cell homeostasis. *Cell*. 161:(2015) 1527–38. doi: 10.1016/j.cell.2015.05.025
130. Song M, Sandoval TA, Chae C-S, Chopra S, Tan C, Rutkowski MR, et al. IRE1α-XBP1 controls T cell function in ovarian cancer by regulating mitochondrial activity. *Nature*. (2018) 562:423–8. doi: 10.1038/s41586-018-0597-x
131. Ma X, Bi E, Lu Y, Su P, Huang C, Liu L, et al. Cholesterol induces CD8+ T cell exhaustion in the tumor microenvironment. *Cell Metab*. (2019) 30:143–156.e145. doi: 10.1016/j.cmet.2019.04.002
132. Thevenot PT, Sierra RA, Raber PL, Al-Khami AA, Trillo-Tinoco J, Zarreii P, et al. The stress-response sensor chop regulates the function and accumulation of myeloid-derived suppressor Cells in tumors. *Immunity*. (2014) 41:389–401. doi: 10.1016/j.immuni.2014.08.015
133. Mehlen P, Puisieux A. Metastasis: a question of life or death. *Nat Rev Cancer*. (2006) 6:449–58. doi: 10.1038/nrc1886
134. Steeg PS. Targeting metastasis. *Nat Rev Cancer*. (2016) 16:201–18. doi: 10.1038/nrc.2016.25
135. Fischer KR, Durrans A, Lee S, Sheng J, Li F, Wong STC, et al. (2015). Epithelial-to-mesenchymal transition is not required for lung metastasis but contributes to chemoresistance. *Nature*. (2015) 527:472–76. doi: 10.1038/nature15748
136. Aielli F, Ponzetti M, Rucci N. Bone metastasis pain, from the bench to the bedside. *Int J Mol Sci*. (2019) 20:280. doi: 10.3390/ijms20020280
137. Wang D, Dubois RN. Eicosanoids and cancer. *Nat Rev Cancer*. (2010) 10:181–93. doi: 10.1038/nrc2809
138. Sabino MAC, Ghilardi JR, Jongen JLM, Keyser CP, Luger NM, Mach DB, et al. (2002). Simultaneous reduction in cancer pain, bone destruction, and tumor growth by selective inhibition of cyclooxygenase-2. *Cancer Res*. 62:7343–9.
139. Chopra S, Giovanelli P, Alvarado-Vazquez PA, Alonso S, Song M, Sandoval TA, et al. IRE1α-XBP1 signaling in leukocytes controls prostaglandin biosynthesis and pain. *Science*. (2019) 365:eaau6499. doi: 10.1126/science.aau6499
140. Lawson DA, Kessenbrock, K, Davis RT, Pervolarakis N, Werb Z. Tumour heterogeneity and metastasis at single-cell resolution. *Nat Cell Biol*. (2018) 20:1349–60. doi: 10.1038/s41556-018-0236-7
141. Ombrato L, Nolan E, Kurelac I, Mavousian A, Bridgeman VL, Heinze I, et al. Metastatic-niche labelling reveals parenchymal cells with stem features. *Nature*. (2019) 572:603–8. doi: 10.1038/s41586-019-1487-6
142. Mahadevan NR, Rodvold J, Sepulveda H, Rossi S, Drew AF, Zanetti M. Transmission of endoplasmic reticulum stress and pro-inflammation from tumor cells to myeloid cells. *Proc Natl Acad Sci USA*. (2011) 108:6561–6. doi: 10.1073/pnas.1008942108
143. Rodvold JJ, Chiu KT, Hiramatsu N, Nussbacher JK, Galimberti V, Mahadevan N R, et al. Intercellular transmission of the unfolded protein response promotes survival and drug resistance in cancer cells. *Sci. Signal*. (2017) 10:eaah7177. doi: 10.1126/scisignal.aah7177

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NDUFA4L2 Regulated by HIF-1α Promotes Metastasis and Epithelial–Mesenchymal Transition of Osteosarcoma Cells Through Inhibiting ROS Production

Wen-Ning Xu†, Run-Ze Yang†, Huo-Liang Zheng†, Lei-Sheng Jiang and Sheng-Dan Jiang**

Department of Clinic of Spine Center, Xinhua Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

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***Correspondence:**
Sheng-Dan Jiang
jiangshengdan@xinhuamed.com.cn
Lei-Sheng Jiang
jiangleisheng@xinhuamed.com.cn

† These authors have contributed equally to this work

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Osteosarcoma (OS) accounts for a large proportion of the types of bone tumors that are newly diagnosed, and is a relatively common bone tumor. However, there are still no effective treatments for this affliction. One interesting avenue is related to the mitochondrial NDUFA4L2 protein, which is encoded by the nuclear gene and is known to be a critical mediator in the regulation of cell survival. Thus, in this study, we aimed to investigate the effect of NDUFA4L2 upon the metastasis and epithelial–mesenchymal transition of OS. We found that NDUFA4L2 protein expression was upregulated in hypoxic conditions. We also used 2-ME and DMOG, which are HIF-1α inhibitors and agonists, respectively, to assess the effects related to decreasing or increasing HIF-1α expression. 2-ME caused a significant decrease of NDUFA4L2 expression and DMOG had the opposite effect. It was obvious that down-regulation of NDUFA4L2 had a direct interaction with the apoptosis of OS cells. Western blotting, wound healing analyses, Transwell invasion assays, and colony formation assays all indicated and supported the conclusion that NDUFA4L2 promoted OS cell migration, invasion, proliferation, and the epithelial–mesenchymal transition. During experiments, we incidentally discovered that autophagy and the ROS inhibitor could be used to facilitate the rescuing of tumor cells whose NDUFA4L2 was knocked down. Our findings will help to further elucidate the dynamics underlying the mechanism of OS cells and have provided a novel therapeutic target for the treatment of OS.

Keywords: HIF-1a, hypoxia, osteosarcoma, metastases, NDUFA4L2

INTRODUCTION

Osteosarcoma (OS) accounts for a large proportion of primary malignant bone tumors that significantly affect children, teenagers, and young adults, accounting for 20–35% of all such diagnoses (Torre et al., 2015). Although there are many treatments that have emerged in recent years, such as chemotherapy and effective resection, the 5-year overall survival rate of OS remains poor, mainly as a result of metastases and relapse (Harrison et al., 2018). The underlying mechanisms inducing the evolution and progression of OS remain poorly

understood. Consequently, it is of significance for the research community to elucidate the potential mechanisms of OS and to facilitate the discovery of novel and effective treatment approaches.

The oxygen concentration of normal non-diseased tissue is about 4%, while the oxygen concentration of solid tumorous tissues is <1% (Muz et al., 2015). OS cells usually survive in low-oxygen conditions, and this advantage plays an important role in the rapid rates of tumor proliferation (Ebbesen et al., 2004). Cells of solid tumors that have survived in a hypoxic environment can activate hypoxic gene pathways and accelerate tumor chemoresistance, which eventually causes a poor prognosis (Keith and Simon, 2007). Hypoxia inducible factor-1 (HIF-1) protein can regulate hypoxic genes of cancer cells and thus can help cope with the hypoxic environments. Notably, HIF-1 protein is comprised of a hypoxia-regulated α subunit and a non-hypoxia-regulated β subunit (Goda et al., 2003). In a normoxic environment, HIF-1α is hydroxylated by prolyl hydroxylases, and hydroxylated HIF-1α is eventually disintegrated by the proteasome. Activated prolyl hydroxylases can be inhibited when cancer cells are exposed to hypoxic environments and when they become hypoxic (Huang et al., 1998; Maxwell et al., 1999; Jaakkola et al., 2001).

NDUFA4L2, a component of the electron transport chain (ETC) complex I subunit, is highly expressed in hypoxic environments, and plays an important role in fine adjustments of complex I activity. Consequently, NDUFA4L2 can mediate the function of oxidative phosphorylation and reactive oxygen species (ROS) production in mitochondria. At present, little is known with respect to the function of NDUFA4L2, especially in regards to its possible functions and roles in OS development. NDUFA4L2 was knocked out in cells, which survived in a hypoxic environment and subsequently promoted mitochondrial ROS production (Tello et al., 2011). This indicated that the NDUFA4L2 protein repressed ROS production and consequently induced anti-oxidative stress in cancer cells (Tello et al., 2011). DNA destruction induced by high ROS accumulation is also known to have detrimental effects upon the survival, proliferation, and metastasis of cancer cells. A recent study reported that NDUFA4L2 accelerated the survival of Non-small cell lung cancer (NSCLC) in hypoxic conditions (Meng et al., 2019). However, the underlying mechanisms of how NDUFA4L2 appears to control and influence the survival of OS is unknown.

Autophagy, a highly conserved biological process, disposes of abnormal or misfolded proteins and limits dysregulation as well as unnecessary organelles in a lysosome-dependent manner (Weckman et al., 2015; Mowers et al., 2017). In recent decades it has emerged that dysfunction of autophagy induced the pathogenesis of varied types of neural diseases (Umezawa et al., 2018), metabolic defects, and the onset of tumors (Huang et al., 2017). However, the specifics of interactions between autophagy and NDUFA4L2 in OS are currently unknown. Therefore, in our study, we sought to explore the influence of NDUFA4L2 and autophagy to the pathogenesis of OS.

MATERIALS AND METHODS

Cell Lines and Cell Culture

The human OS cell lines 143b, HOS, and U2OS were purchased from ATCC Company (Manassas, VA), and were cultured in DMEM modified medium (Gibco, Invitrogen). All medium contained 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were cultured in a consistent and humidified atmosphere of 5% CO_2 at 37°C. Cells were cultured in hypoxic environments (1% O_2) until 70% density.

Cell Proliferation Assay

We used the Cell Counting Kit-8 (CCK-8; Beyotime, Beijing, China) to measure cell viability. 143b, HOS, and U2OS cells were adjusted to 1×10^5 cells/well and were seeded in six-well plates. 24, 48, 72 and 96 h post-transfection, 20 μL of CCK-8 solution was added per well. After 4 h of incubation, cell proliferation was determined by measuring optical density (OD) values at 450 nm on a Microplate Reader (Thermo Fisher Scientific).

Colony Formation Assays

Colony formation assays were performed following previously published methods (Cai et al., 2017). Transfected cells during logarithmic phases were plated in six-well plates at a density of 1,000 cells per well. Two weeks later, we twice washed the cells by using phosphate buffered saline (PBS), and then fixed the samples by using methanol for 30 min. Colonies were then stained by using 0.1% crystal violet (Sinopharm Chemical Reagent, Shanghai, China), and the numbers of colonies were manually calculated.

Flow Cytometry to Detect Cell Apoptosis

Flow cytometry was used to measure the rate of apoptosis by using the Annexin V-FITC Apoptosis Detection Kits (BD Biosciences, Franklin Lakers, NJ) according to the manufacturer's protocol. Cells were incubated with the mixing solution at room temperature for 15 min, and the cells were analyzed by using the FACS System (BD Biosciences).

Immunofluorescence

Cells were seeded on glass coverslips and then fixed with 4% paraformaldehyde for 30 min. The cells were permeabilized in 0.1% Triton X-100 for 20 min. Next, cells were incubated with primary antibodies overnight at 4°C as follows: E-cadherin (1:100, Abcam, United States) and Vimentin (1:200, Abcam, United States). Coverslips were thrice washed with PBST after they were incubated with secondary antibody for 1.5 h at 37°C (Beyotime, China). Immunofluorescence was captured through photography and by using fluorescence microscopy (Olympus BX51).

Western Blotting Assays

The treated cells were harvested and lysed using RIPA buffer (with protease inhibitors). Nuclear protein was harvested by using Nuclear protein and cytoplasmic protein extraction

kit (Beyotime, China). Cell lysates were separated on SDS-PAGE, transferred onto PVDF membranes, blocked with 5% BSA, incubated with primary antibodies against the following: HIF-1α (1:1,000, Proteintech, China), HIF-2α (1:1,000, abcam, United Kingdom), NDUFA4L2 (1:1,000, Proteintech, China), Cytochrome c (1:1,000, Proteintech, China), P62 (1:1,000, Proteintech, China), Beclin-1 (1:1,000, CST, United States), LC3(1:1,000, Novus, United States), Bax (1:1,000, Proteintech, China), Bcl-2 (1:1,000, Proteintech, China), PARP (1:1,000, CST, United States), E-cadherin (1:1,000, CST, United States), Vimentin (1:1,000, CST, United States), Slug (1:1,000, CST, United States), Snail (1:1,000, CST, United States), MMP2 (1:1,000, CST, United States), MMP9 (1:1,000, CST, United States), Lamin B (1:1,000, Beyotime, China), Tubulin (1:1,000, Beyotime, China) and GAPDH (1:1,000, Beyotime, China). Subsequently, membranes were incubated with secondary antibodies and measures were determined using EasyBlot ECL kits (Sangong, Songjiang, Shanghai, China). Membranes were thrice washed with TBST and were then incubated with HRP-conjugated secondary antibodies. Finally, membranes were induced to react with the addition of ECL Plus reagent (Millipore). Results were quantified by Image-J (National Institutes of Health).

RNAi, pcNDA, and Lentivirus Transfection

NDUFA4L2-siRNAs and respective negative control (NC) siRNAs were designed, synthesized, and purchased from GenePharma, China. siRNAs sequences are listed as follows: 5′-CUGAUGACCAGCAACUUGAdTdT-3′ (sense); 5′-UCAAGUUGCUGGUCAUCAGdTdT-3′ (antisense) for si-HIF-1α-1; 5′-GGGCCGUUCAAUUUAUGAATT-3′ (sense) and 5′-GCCUCUUCGACAAVCUUAATT-3′ (antisense) for si-HIF-1α-2; 5′-CAGCAUCUUUGAUAGCAGUdTdT-3′ (sense) and 5′-ACUGCUAUCAAAGAUGCUGdTdT-3′ (Antisense) for si-HIF-2α-1; 5′-CACCGCCGTACTGTCAACCTCAAGTTTCAAGAGAACTT GAGGTTGACAGTACGGCTTTTTTG-3′ (sense) and 5′-GATCCAAAAAAGCCGTACTGTCAACCTCAAGTTCTCTT GAAACTTGAGGTTGACAGTACGGC-3′ (Antisense) for si-HIF-2α-2; 5′-UCCUCGGUACGUGUCACGUTT-3′ (sense) and 5′-ACGUGCCACGAUCGCAGAUTT-3′ (antisense) for si-NC; 5′-GCAGUUUCCACUGACUAUATT-3′ (sense) and 5′-UAUAGUCAGUGGAAACUGCTT-3′ (anti-sense) for si-NDUFA4L2-1; 5′-UCAUCCCGAUGAUCGGCUUTT-3′ (sense) and 5′-AAGCCGAUCAUCGGGAUGATT-3′ (anti-sense) for si-NDUFA4L2–2; 5′-GCUGGGACAGAAAGAACAATT-3′ (sense) and 5′-UUGUUCUUUCUGUCCCAGCTT-3′ (anti-sense) for si-NDUFA4L2–3. pcDNA-NDUFA4L2 plasmids were designed and synthesized chemically (Sangon Biotech, China). The cloning vector was pcDNA3.1+. As described above, NDUFA4L2 plasmids were added to serum-free medium, and then, Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was added to the same medium. The Lentivirus containing shNDUFA4L2 or shNC were transfected into OS cells. After 48 h, cells were used in follow-up experiments.

Chromatin Immunoprecipitation (ChIP) Assays

We used SimpleChIP Enzymatic Chromatin IP kit (Magnetic Beads; CST, Pudong, Shanghai, China) to conduct the ChIP assays following all manufacturer protocols. The precipitated protein/DNA complexes were separately immunoprecipitated with the use of antiSP1 antibody (Abcam, Pudong, Shanghai, China) and IgG antibody (Abcam, Pudong, Shanghai, China). The precipitated DNA was then analyzed in quantitative real time polymerase chain reaction (qRT-PCR). Primer Sequences for CHIP Assays were as follows: Forward: 5′–CAGGTCTGTGTATGTGTGAAA–3′, and Reverse: 5′–CTACGCACTGTCACTGAG–3′.

Transwell Assays

Transwell invasion assays were performed to assess cell invasion. Upper chambers were coated with Matrigel (Corning, NY) and then incubated overnight before cells were plated. Transfected cells were cultured in upper chambers with serum-free medium. In the lower chambers, DMEM with 10% FBS was added. Post 24 h of incubation, remaining non-invaded cells were carefully wiped away. Finally, invaded cells were stained with 0.1% crystal violet (Sinopharm Chemical Reagent). Invaded cells were counted under light microscopy (Olympus, Tokyo, Japan).

Migration Assays

To measure cell migratory ability, 143b were seeded onto a 6 cm plate overnight in a consistent and humidified atmosphere of 5% CO_2 at 37°C. Confluent monolayers were scratched using sterile pipette tips followed by several washes with phosphate-buffered saline (PBS) to remove detached cells. Cells were then transfected with siRNA for 48 h in medium without serum. Photographs of wounded areas were obtained using a Leica DMI3000 B inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany). The migration rate was calculated according to the scratched surfaces, which were quantified using ImageJ Version 1.410 software (National Institutes of Health, Bethesda, MD, United States).

Tumor Xenograft Model

Immunodeficient male BALB/C nude mice (18–20 g) were obtained from the Animal Center of Shanghai Jiao Tong University. Animals were cared for at least 1 week before initiation of experimental phases. Animals were fed with rodent laboratory chow and water *ad libitum* under standard laboratory animal conditions (25°C, 50–70% humidity, 12 h light/dark cycle). Each nude mouse (Five mice per treatment group) was injected subcutaneously with 143b cells (100 μ, 1×10^6) transfected with LV-shNC or LV-shNDUFA4L2. After 2 weeks, mice were sacrificed and tumors were excised. Tumors were weighed as well as subjected subsequently to immunohistochemical assays. All experiments were carried out in accordance with Guidelines for the Care and Use of Laboratory Animals and approved by the Xinhua Hospital, Shanghai Jiao Tong University School of Medicine.

Immunohistochemical Examination

Immunohistochemical examinations were performed following methods outlined in a previous study. Briefly, antigen was retrieved and microwaved for 15 min. Next, endogenous peroxidase activity was blocked for 10 min by use of 3% hydrogen peroxide, and then non-specific binding sites were blocked for 30 min at room temperature by 5% BSA (bovine serum albumin). Primary antibodies were added to sections and incubated overnight at 4°C. For primary antibodies we used anti-LC3, 1:100, Novus, United States, and (PCNA, 1:100, Proteintech, China). Sections were incubated with an appropriate HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Dallas, TX, United States) and counterstained with hematoxylin.

ROS Measurement

Mitochondrial ROS production was detected by Reactive Oxygen Species Assay Kit (Beyotime, China) according to the manufacturer's protocol. After various treatments, Os cells were washed with PBS and incubated with serum-free medium containing with DCFH-DA at 37°C for 20 min. Then DCFH-DA was removed and washed with serum-free medium three times. DCF fluorescence distribution of cells was detected using fluorescence microscope analysis (Olympus Fluoview, Japan). Positive cells were emitted with green.

TUNEL Assays for Apoptosis

OS cell apoptosis was determined using One-step TUNEL cell apoptosis detection kits (Beyotime, No. C1086, Shanghai, China) following manufacturer protocols. OS cells were seeded upon coverslips in six-well plates. Post-application of varied treatments, cells were washed using PBS. Cells were then fixed with 4% paraformaldehyde for 30 min and then washed once with PBS. Cells were permeabilized with PBS containing 0.3% Triton X-100 at room temperature for 5 min. Cells were then twice washed with PBS. We then added 50 μL of TUNEL detection solution to samples and incubated at 37°C for 60 min in the dark. Finally, we added DAPI, incubated for 5 min, and completed imaging using fluorescence microscopy (Olympus BX51).

Oxygen Consumption

Oxygen consumption (OCR) was measured by high-resolution respirometry (Oxygraph-2k, Orobros Instruments, Innsbruck, Austria). After OS cells were trypsinized, they were resuspended in HBSS containing 25 mM HEPES at 2×10^6 cells/ml. The instrument background flux was calculated as a linear function of oxygen concentration, and the experimental data were corrected using DatLab software (Oroboros Instruments). The oxygen concentration in the air saturated medium was 175.7 μM at 37°C. The oxygen concentration of cells was measured in a 37°C box under normoxic and hypoxic (1% O2) environments with the indicated treatments.

Lactate Production

To evaluate the production of lactate, a lactic acid assay kit (BioVision) was used to explore the cell culture medium according to the manufacturer's protocol. These values were normalized to protein concentration.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism 5. All data were presented as the mean ± standard deviation (SD). Independent-sample *t*-tests were performed to facilitate analysis of differences between treatments. For multiple comparisons, a two-way analysis of variance (ANOVA) was performed followed by a Tukey *post-hoc* test. All p-values were two-sided and nsp ≥ 0.05, $^{*}p < 0.05$, $\psi p < 0.01$, and $\#p < 0.001$ were deemed as levels of statistical significance. All experiments were replicated three times.

RESULTS

HIF-1α Can Regulate NDUFA4L2 Expression in 143b and U2OS in Hypoxic Conditions

To investigate hypoxia adaptation mechanisms in human OS, we performed Western blotting and characterized levels of gene expression of two human OS cell lines, 143b and U2OS. Cells were cultured in normoxic and hypoxic environments for 24 and 48 h, respectively. The results for 143b and U2OS indicated that HIF-1α and NDUFA4L2 expression was significantly upregulated under hypoxic conditions (**Figures 1A,B**). We further examined the Cytochrome C and autophagy relative protein, p62, Beclin-1, LC3, and found that the activity of autophagy was much higher under hypoxic vs. non-hypoxic conditions (**Figure 1A**). In hypoxic environments, Cytochrome c was released to cytoplasm from mitochondria, indicating that there was some apoptosis in OS cells cultured in hypoxic environments (**Supplementary Figure S1A**). The following experiments were performed in hypoxic environments for 48 h. The ROS production was also increased in hypoxic environments (**Figure 1B**). The lower OCR and higher lactate production demonstrated that OS cells mainly used glycolysis to produce energy in hypoxic environments (**Supplementary Figures S1B,C**). An autophagy indicator was used to track autophagy flux, and results indicated that autophagy flux was significantly enhanced under hypoxic environments (**Figure 1C**). Thus, we inferred there were important connections among NDUFA4L2, autophagy, and apoptosis.

Treatment of cells with HIF-1α inhibitor (2-methoxyestradiol, 2-ME, 20 mmol/L) (Chen et al., 2015) and agonist (Dimethyloxaloylglycine, DMOG, hydroxylase inhibitor, 0.2 mmol/L) (Xie et al., 2012), respectively was performed. Western blotting results revealed that suppression of HIF-1α induced reductions in NDUFA4L2 and that accelerated HIF-1α expression could heighten NDUFA4L2 protein expression compared to the control group (**Figure 1D**). CHIP assays were performed on 143b cells. To determine the level of consequence that HIF-1α bound to NDUFA4L2' HREs, qPCR was carried out with specific primer for attachment to the HRE site. Schematic representations of the human NDUFA4L2 gene and the nucleotide sequences matching HRE from six

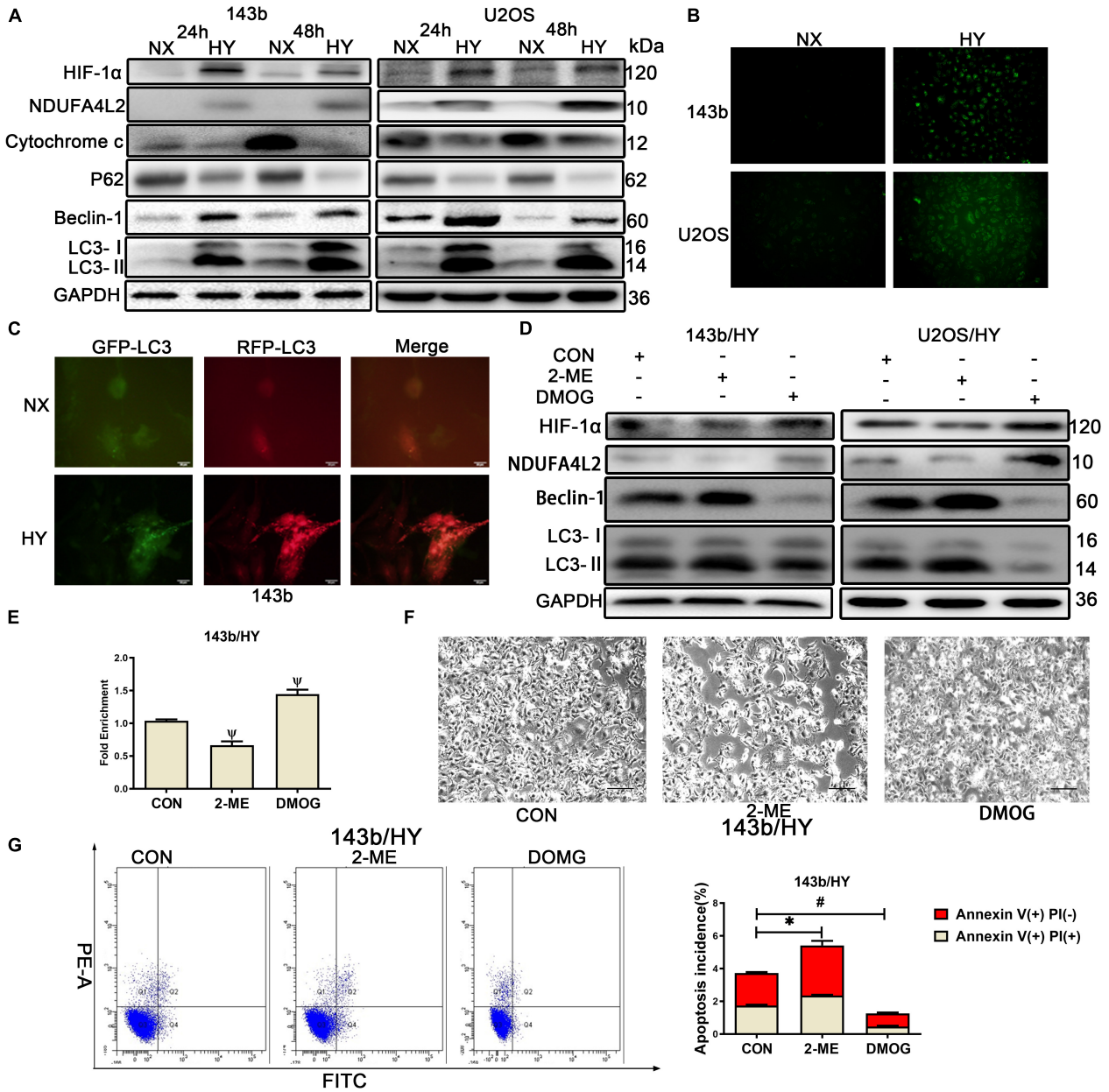

FIGURE 1 | HIF-1α might regulate the expression of NDUA4L2 in 143b and U2OS cell lines. 143b and U2OS cells were cultured in hypoxic environments for 24 and 48 h while control cells were cultured in normoxic environments. **(A)** Protein expression of HIF-1α, NDUFA4L2, P62, Beclin-2, LC3, and GAPDH in 143b and U2OS cells was determined by Western blotting. **(B)** ROS production was detected in 143b and U2OS under normoxic environments or hypoxic environments for 48 h by use of a Reactive Oxygen Detection Kit. **(C)** Fluorescence-based imaging for 143b cells transfected with mRFP-GFP-LC3 adenovirus. Green dot represents the start of autophagic flux, and Red dot represents the end of autophagic flux. Promotion of Red dot represents that autophagic flux was promoted. 143b cells were under normoxic environments or hypoxic environments for 48 h. **(D)** Expression of HIF-1α, NDUFA4L2, Beclin-1, LC3, and GAPDH were determined by Western blotting in 143b and U2OS cells pretreated with 2-ME and DMOG in hypoxic environments. **(E)** CHIP assay of HIF-1α binding to NDUFA4L2 gene in 143b cells. **(F)** About 1×10^6 143b cells were planted into six-well plates and cultured at 37°C until 70% confluence. 143b cell morphology was photographed using microscopy. Scale Bar = 100 μm. **(G)** Apoptosis of 143b cells was detected by flow cytometry. NX, normoxic environment; HY, hypoxic environment. nsp ≥ 0.05, *p < 0.05, ψ < 0.01, and # < 0.001 were defined as measures that indicated significant differences among treatment groups. All experiments were performed in triplicate.

mammalian genes was provided in **Supplementary Figure S1D**. The level of consequence that HIF-1αbound to NDUFA4L2' HREs was found to have decreased in the 2-ME treatment group and contrastingly increased in the DMOG treatment group (**Figure 1E**). 143b cells that were treated with HIF-1α agonist were found to have had vigorous growth compared to the control group as characterized under light microscopy. However, we found opposite results in the treatment of 143b

cells with 2-ME (**Figure 1F**). The cell proliferation detected by CCK-8 confirmed that HIF-1(α inhibitor 2-ME repressed the cell proliferation of OS cells and HIF-1(α agonist DMOG promoted the cell proliferation of OS cells in hypoxic environments (**Supplementary Figure S1E**). Importantly, the incidence of apoptosis was detected by use of flow cytometry Annexin V/PI double staining. Results confirmed that 143b cells treated with DMOG had lower apoptosis incidence (**Figure 1G**). However, the very low percentages of dead cells seemed to represent a normal rate of cell death rather than actually increased apoptosis. Therefore, we pretreated the cells with staurosporine (100 nM) (Lien et al., 2018) to quantify the relative effect of DMOG and ME treatments upon dead cells. The results confirmed the above findings that OS cells treated with DMOG had a lower incidence of apoptosis (**Supplementary Figure S1F**). We further examined autophagy relative proteins, Beclin-1 and LC3. We found that autophagy activity was significantly inhibited when DMOG was added to 143b and U20S, and autophagy activity was significantly promoted when 2-ME was added to the OS cell lines (**Figure 1D**).

To further confirm the relationship between HIF-1α and NDUFA4L2, small interfering RNAs to HIF-1α and HIF-2α were established to facilitate silencing of expression HIF-1α and HIF-2α. The effect of si-HIF-1α and si-HIF-2α in hypoxic environments was confirmed by use of qRT-PCR and Western blotting. HIF-1α protein expression was knocked down by si-HIF-1(α-1 and si-HIF-1(-2 significantly (**Supplementary Figure S1G**). HIF-1α knockdown induced decreased expression of NDUFA4L2 protein in OS cells (**Supplementary Figure S1G**). CHIP assays indicated that HIF-1α bound to NDUFA4L2' HREs was decreased by way of si-HIF-1α (**Supplementary Figure S1I**). Moreover, we found that HIF-2α knockdown by si-HIF-2α-1 and si-HIF-2α-2 did not induce a reduction in expression of NDUFA4L2 and HIF-2α bound to NDUFA4L2' HREs did not decreased by way of si-HIF-2α (**Supplementary Figures S1H,J**). These results revealed that HIF-1α regulated expression of NDUFA4L2 and regulated levels of autophagy in hypoxic environments.

Knockdown of NDUFA4L2 Inhibits Osteosarcoma Cell Proliferation, Migration, and Epithelial–Mesenchymal Transition Progression and Promotes Apoptosis *in vitro*

We found that NDUFA4L2 expression was increased, but also found that Cytochrome c expression was decreased in mitochondria of OS cells under hypoxic environments. Consequently, to examine if NDUFA4L2 could inhibit apoptosis in OS cell lines cultured in hypoxic environments, small interference including si-NC, si-NDUFA4L2-1, si-NDUFA4L2-2 and si-NDUFA4L2-3 was used to knock down NDUFA4L2 in 143b, U2OS, and HOS cells cultured under hypoxic environments. Western bolting results showed that NDUFA4L2 was silenced significantly by si-NDUFA4L2-1, si-NDUFA4L2-2, and si-NDUFA4L2-3 (**Supplementary Figure S2A**). Bcl-2/Bax has a negative correlation with apoptosis incidence. The more apoptosis happens, the greater ratio of C-PARP/T-PARP is observed in apoptosis cells. **Figure 2A** demonstrated that knockdown of NDUFA4L2 induced decreased expression of Bcl-2/Bax and increased expression of C-PARP/T-PARP compared to the control group in 143b, U2OS, and HOS cell lines. Flow cytometry Annexin V/PI double staining and TUNEL assays demonstrated that si-NDUFA4L2 promoted apoptosis of HOS and 143b cells (**Supplementary Figure S2C**). At the cellular level, we used CCK-8 to examine relative cell proliferation whereby results suggested that proliferation was reduced in cells whose NDUFA4L2 was knocked down (**Figure 2B** and **Supplementary Figure S2B**). Furthermore, silencing of NDUFA4L2 induced production of ROS (**Figure 2C**). Intriguingly, autophagy was increased in these cells transfected with si-NDUFA4L2 (**Figure 2A**). We inferred that mitochondrial NDUFA4L2 might be essential for OS cells survival. However, the role of autophagy still remains unknown and was elucidated in our study.

To assess if NDUFA4L2 regulated cancer metastasis in OS cells, we established small interference RNA to silence NDUFA4L2. The results of associated wound healing assays suggested that 143b and HOS cells transfected with si-NDUFA4L2 had reduced migration related abilities (**Supplementary Figure S2F**). At the molecular level, results of immunofluorescence and Western blotting indicated that there were increases in epithelial–mesenchymal transition (EMT) relative protein E-cadherin expression, and decreases in EMT relative protein Vimentin in OS cells transfected with si-NDUFA4L2 (**Figure 2E** and **Supplementary Figure S2E**). Silencing of NDUFA4L2 also induced an increase in measures of E-cadherin, and induced a decrease in Vimentin, Slug, Snail, and MMP9 expression (**Figure 2D**). However, MMP2 protein expression was increased in cells transfected with si-NDUFA4L2. Importantly, NDUFA4L2 knockdown led to an increased OCR in OS cells under hypoxic environments (**Figure 2F**). Finally, we established pcDNA-NDUFA4L2 to overexpress NDUFA4L2 expression in HOS cells cultured in normoxic environments. Our results showed that overexpression of NDUFA4L2 in normoxia was insufficient alone to activate the EMT progression (**Figure 2G**). These results revealed that mitochondrial NDUFA4L promoted OS cell migration and EMT progression in hypoxic environments.

NDUFA4L2 Protects Osteosarcoma Cells by Repressing ROS Production

A recent study reported that NDUFA4L2 could function as an antioxidant (Lien et al., 2018). To confirm the role of ROS in OS cells post-silencing of NDUFA4L2, we applied NAC (N-acetylcysteine, 10mM) (Li et al., 2017), which is a scavenger of ROS, to treat 143b, U2OS, and HOS cells in hypoxic environments. NAC did not affect the control cells no matter whether or not OS cells were cultured in normal or hypoxic environments (**Supplementary Figures S3A–C**). NDUFA4L2 knockdown did not affect OS cells under normal environments either (**Supplementary Figure S3C**). NDUFA4L2 was downregulated in OS cells cultured in non-hypoxic environments. NDUFA4L2 knockdown did not induce an increase of ROS production and thereby NAC did not affect

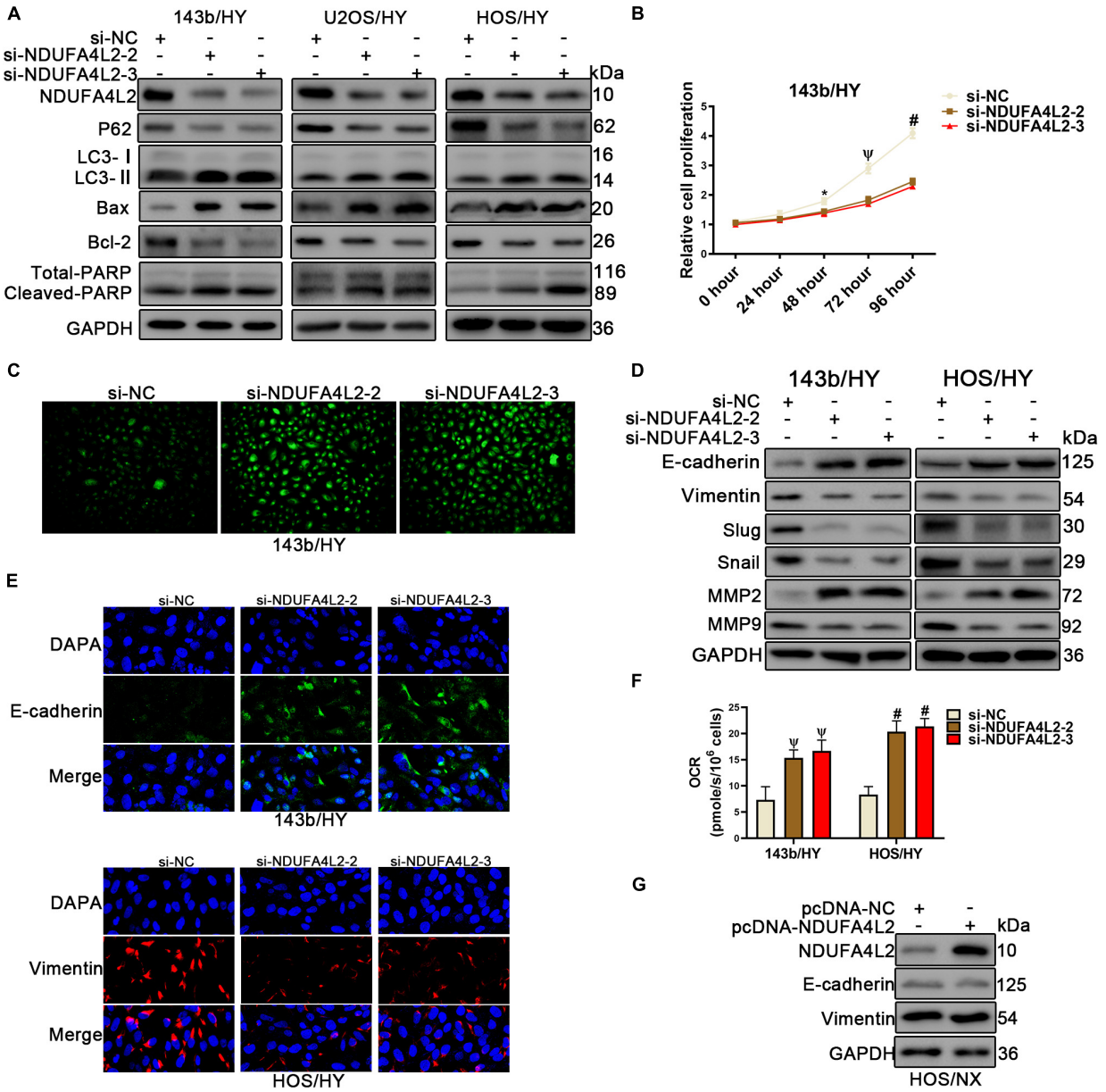

FIGURE 2 | Knockdown of NDUFA4L2 inhibited OS cell proliferation migration, and epithelial–mesenchymal transition progression, as well as promotes apoptosis *in vitro*. 143b, U2OS, and HOS cell lines were transfected with si-NC or si-NDUFA4L2-2 or si-NDUFA4L2-3 in hypoxic environments. **(A)** Protein expression of NDUA4L2, P62, LC3, Bax, Bcl-2, PARP, and GAPDH in 143b, U2OS, and HOS cells was measured using Western blotting. **(B)** Relative cell proliferation of 143b cells was detected by CCK-8. **(C)** ROS production was detected by use of a Reactive Oxygen Detection Kit. **(D)** Protein expression of Slug, snail, MMP2, MMP9, E-cadherin, and Vimentin was measured in 143b and HOS cells post-transfection of si-NC, si-NDUFA4L2-1, or si-NDUFA4L2-2. **(E)** Immunofluorescence assessments were performed to measure E-cadherin and Vimentin protein expression in 143b and HOS cells. **(F)** Protein expression of NDUFA4L2, E-cadherin, and Vimentin was measured in HOS cells. NX, normoxic environment; HY, hypoxic environment. nsp ≥ 0.05, $^{*}p < 0.05$, $\psi < 0.01$, and # < 0.001 were defined as measures that indicated significant differences among treatment groups. All experiments were performed in triplicate. **(F)** OCR were detected in 143b and HOS cells. **(G)** Protein expression of NDUFA4L2, E-cadhrin, and Vimentin was measured in HOS cells.

OS cells cultured in non-hypoxic environments. However, results of Western blotting indicated that NAC attenuated apoptosis and autophagy of OS cells transfected with si-NDUFA4L2 (**Figure 3A**). TUNEL assays confirmed that NAC rescued OS cell apoptosis (**Supplementary Figure S3D**). Ultimately, these results suggested that NAC could rescue the survival of OS cells under a hypoxic environment when NDUFA4L2 was knocked down.

To further investigate whether or not NDUFA4L2 regulated proliferation, invasion, and EMT progression of OS cell lines through the effect of repression of ROS production, we performed colony formation assays, transwell assays, and

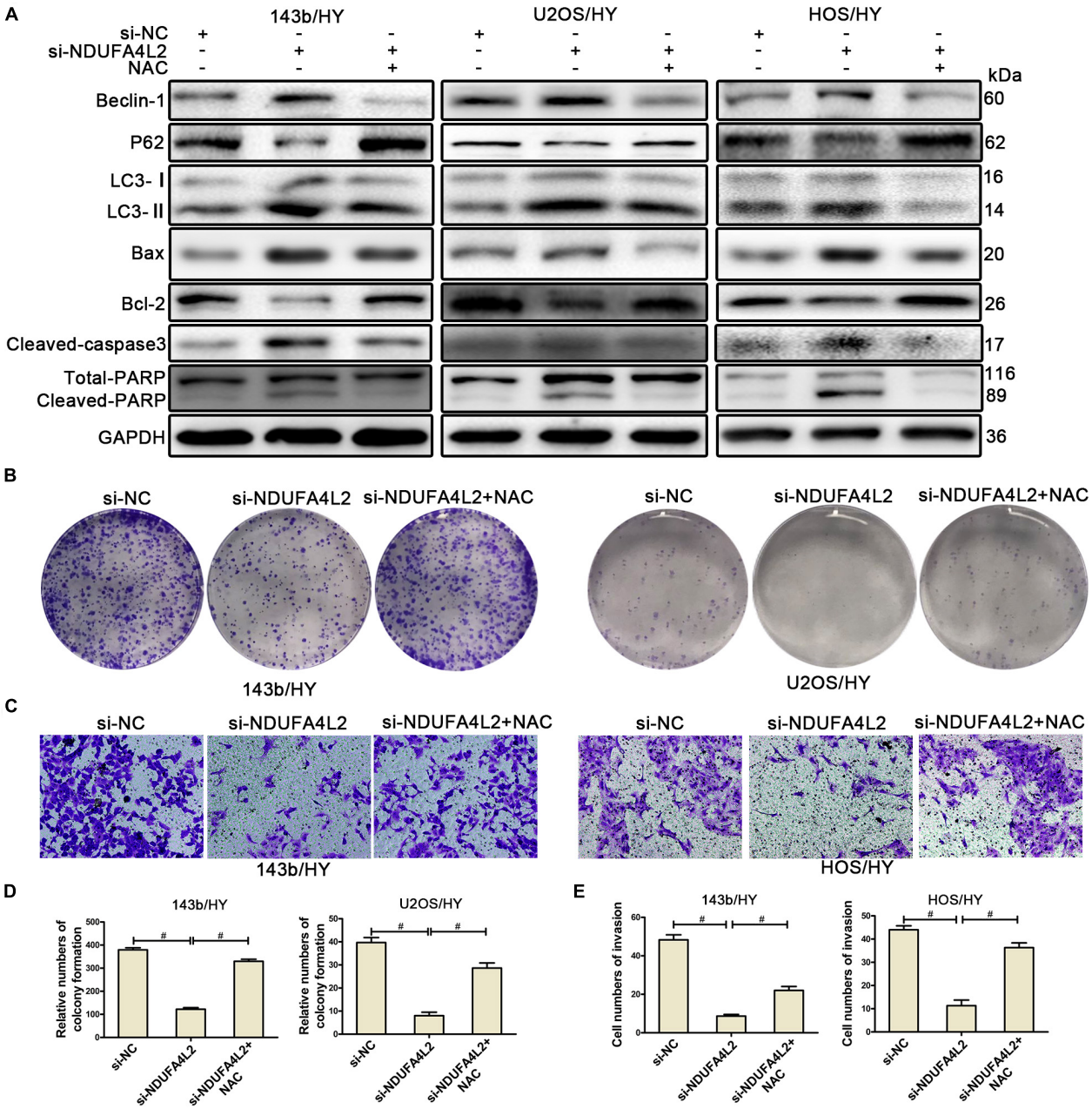

FIGURE 3 | NDUFA4L2 protected OS cells by repressing ROS production. 143b, U2OS, and HOS cells were treated with NAC post-transfection with si-NC, si-NDUFA4L2-1, or si-NDUFA4L2-2 in hypoxic environments. **(A)** Western blotting facilitated determinations of protein expression of Beclin-1, P62, LC3, Bax, Bcl-2, Cleaved-caspase3, PARP, and GAPDH in 143b, U2OS, and HOS cells. **(B,D)** The colonizing ability of si-NDUFA4L2-transfected 143b and U2OS cells post-treatment with NAC was determined by using colony formation assays. **(C,E)** Invasion ability of si-NDUFA4L2-transfected 143b and HOS cells post-treatment with NAC was determined by using transwell assays. HY: hypoxic environment. nsp $\geq$ 0.05, and # $<$ 0.001 were defined as measures that indicated significant differences among treatment groups. All experiments were performed in triplicate.

Western blotting to evaluate the proliferation, invasion, and EMT progression of OS cell lines transfected with si-NDUFA4L2 after treatment with the ROS inhibitor. **Figures 3B,D** indicated that 143b and U2OS cells transfected with si-NDUFA4L2 had a lower degree of colony formation than cells transfected with si-NC, whereas NAC could reverse this effect. We also observed that NAC promoted invasion of 143b and HOS cells transfected with si-NDUFA4L2. Results of transwell assays indicated that knockdown of NDUFA4L2 consequently induced inhibition of the invasion of 143b and HOS, whereas NAC could reverse this effect (**Figures 3C,E**). Furthermore, Western blotting indicated that NAC promoted EMT progression of OS cells

transfected with si-NDUFA4L2 (**Supplementary Figure S3E**). These outcomes indicated that NDUFA4L2 could promote the proliferation, invasion and EMT progression of OS through repressing ROS production.

Autophagy Promotes the EMT Progression, Invasion, Migration, and Proliferation in Osteosarcoma Cell Transfected With si-NDUFA4L2 by Eliminating ROS Production

According to previous research, we speculated that autophagy was positively correlated with metastasis of OS cells. Autophagy protected the survival, metastasis, and EMT progression by removing a large amount of ROS in OS cells when NDUFA4L2 was repressed. To confirm this role of autophagy in OS cells cultured in hypoxic environments, Rapamycin (10 nM) (Li et al., 2019) was used to treat 143b and HOS after transfection with si-NDUFA4L2. As demonstrated in **Figure 4A**, protein expression of P62 decreased and LC3 increased in si-NC + Rapamycin, si-NDUFA4L2 and si-NDUFA4L2 + Rapamycin based treatment groups. Compared with si-NC + Rapamycin and si-NDUFA4L2 groups, the trend is more obvious in si-NDUFA4L2 + Rapamycin groups. Thus, these results confirmed that Rapamycin promoted autophagy. Subsequently, we found that treatment with rapamycin alone did not significantly change E-cadherin and Vimentin protein expression. However, Rapamycin was capable of enhancing the protein-based expression of E-cadherin and was capable of reducing Vimentin expression after silencing of NDUFA4L2 (**Figure 4A**). Notably, immunofluorescence confirmed all of our above findings (**Figure 4B** and **Supplementary Figures S3F,G**). Subsequent to these findings, we next investigated the role of autophagy in the invasion of OS cells transfected with si-NDUFA4L2 by using transwell assays. **Figure 4C** indicated that autophagy also enhanced the invasion of OS cells transfected with si-NDUFA4L2. These results indicated that autophagy promoted the progression of EMT and invasion of OS cells under a hypoxic environment when NDUFA4L2 was repressed.

To confirm the role of autophagy in migration and proliferation, we used wound healing analysis and colonizing assays for OS cells treated with Rapamycin after transfected with si-NDUFA4L2. As was demonstrated in **Supplementary Figure S4A**, the migration of HOS cells was repressed, and autophagy was capable of promoting the migration of HOS transfected with si-NDUFA4L2. Furthermore, the results from colony formation assays demonstrated that autophagy could enhance the proliferation of OScells post-transfection with si-NDUFA4L2 (**Supplementary Figure S4B**). However, treatments with Rapamycin alone did not significantly change the migration and cloning ability of OS cells (**Supplementary Figures S4A,B**). To further assess whether or not autophagy regulated metastasis and epithelial–mesenchymal transition of osteosarcoma cells though the removal of ROS production, we assessed ROS levels by using Reactive Oxygen Species Assay Kits. The corresponding results demonstrated that Rapamycin reduced ROS levels in OS cells transfected with si-NDUFA4L2 and treatments with Rapamycin alone could also decrease the ROS levels in hypoxic environments (**Supplementary Figure S4C**). These results indicated that treatments with Rapamycin alone were able to reduce the ROS levels while they did not significantly promote the invasion, migration, proliferation and EMT ability of OS cells. Rapamycin could significantly promote the invasion, migration, proliferation and EMT ability of OS cells transfected with si-NDUFA4L2 in hypoxic environments.

To further verify the role of autophagy in OS cells under hypoxic environments when NDUFA4L2 was inhibited, Chloroquine (CQ, 10 μM) (He et al., 2019) was used to treat OS cells transfected with si-NDUFA4L2. As was demonstrated in **Figure 5A**, CQ caused an increased level of P62 and LC3, which showed autophagy was inhibited, and was likely repressed by CQ. Next, Western blotting and transwell assays were used to assess the effect of autophagy in OS cells. **Figure 5A** indicated that EMT progression in OS cells were decreased due to the knockdown of NDUFA4L2, and furthermore suggested that CQ enhanced this effect. Additionally, **Figure 5B** demonstrated that restraint of autophagy further inhibited the invasion of OS cells induced by knockdown of NDUFA4L2. Interestingly, ROS production in OS cells increased (**Figure 5C**). Importantly, treatments with CQ alone were able to remarkably inhibit EMT and invasion of OS cells as well as increasing ROS levels in hypoxic environments (**Figures 5A–C**). These results revealed that autophagy promoted EMT progression, invasion, migration, and proliferation in OS cells transfected with si-NDUFA4L2 by eliminating ROS production.

NDUFA4L2 Knockdown Inhibits Osteosarcoma Growth *in vivo*

The function of NDUFA4L2 *in vivo* was evaluated in BALB/c nude mice xenografted with 143b cells. The effect of Lenti-shNDUFA4L2 was confirmed by qRT-PCR and Western blotting. The levels of mRNA and protein expression of NDUFA4L2 in 143b cells were reduced significantly (**Figures 6A,B**). There was a significant decrease in tumor volume in the Lenti-shNDUFA4L2 treatment group (**Figure 6C**). It can be seen in **Figure 6D** that tumor volume was decreased obviously at time steps of 7, 10, and 15 days. Furthermore, tumor weight, PCNA and LC3-*II* were measured. Findings indicated that compared with the Lenti-NC group, tumor weights in the Lenti-NDUFA4L2 group were significantly reduced and expression of PCNA in the Lenti-shNDUFA4L2 group was significantly downregulated in OS tissues derived from nude mice. LC3-*II* protein expression was upregulated (**Figure 6E**). However, there were no significant differences in HIF-1α expression between Lenti-NC and Lenti-shNDUFA4L2 groups (**Supplementary Figure S5A**), confirming that NDUFA4L2 knockdown did not change the expression level of HIF-1α and HIF-1α regulate NDUFA4L2 expression. Moreover, HIF-1α was accumulated in the nuclei of cancer cells *in vivo* (**Supplementary Figures S5A,B**). These results confirmed that knockdown of NDUFA4L2 induced the inhibition of growth of OS tumors and that autophagy could be induced when mitochondrial NDUFA4L2 was silenced.

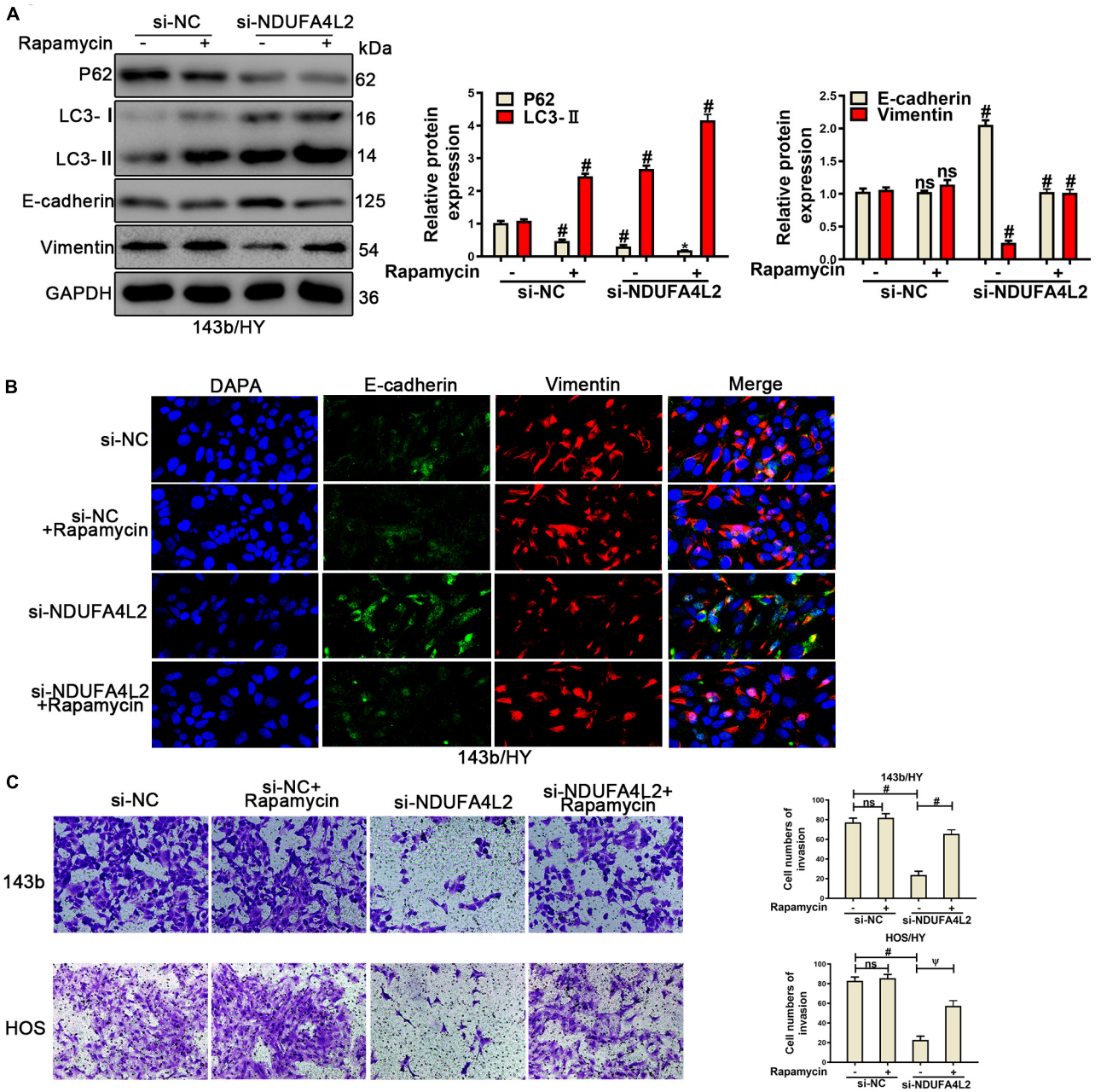

FIGURE 4 | Upregulation of autophagy promoted the invasion and EMT progression of si-NDUFA4L2-transfected 143b and HOS cells. 143b and HOS cells were treated with Rapamycin post-transfection with si-NC or si-NDUFA4L2 in hypoxic environments. **(A)** Western blotting was performed to determine protein expression of P62, LC3, E-cadherin, and Vimentin in 143b cells. For P62, si-NC vs. si-NC + Rapamycin was #$p < 0.001$, si-NC vs. si-NDUFA4L2 was #$p < 0.001$, si-NDUFA4L2 vs. si-NDUFA4L2 + Rapamycin was *$p < 0.05$; For LC3, si-NC vs. si-NC + Rapamycin was #$p < 0.001$, si-NC vs. si-NDUFA4L2 was #$p < 0.001$, si-NDUFA4L2 vs. si-NDUFA4L2 + Rapamycin was #$p < 0.001$; For E-cadherin and Vimentin, si-NC vs. si-NC + Rapamycin was nsp ≥ 0.05, si-NC vs. si-NDUFA4L2 was # < 0.001, si-NDUFA4L2 vs. si-NDUFA4L2 + Rapamycin was # < 0.001. **(B)** Immunofluorescence was performed to measure E-cadherin and Vimentin expression in 143b cells. **(C)** Transwell assays were performed to determine the invasion ability of si-NDUFA4L2-transfected 143b and HOS cells post-treatment with Rapamycin. HY: hypoxic environment. nsp ≥ 0.05, $\psi < 0.01$, and # < 0.001 were defined as measures that indicated significant differences among treatment groups. All experiments were performed in triplicate.

To confirm the role of NAC in OS tumors transfected with Lenti-shNDUFA4L2, we treated BALB/c nude mice with NAC (7 mg/mL). We found that NAC facilitated the growth of OS tumors (**Supplementary Figure S5C**). NAC increased tumor volume and weight in the Lenti-shNDUFA4L2 + NAC treatment group (**Supplementary Figures S5D,E**). PCNA expression increased and LC-3 expression decreased significantly in the Lenti-shNDUFA4L2 + NAC treatment group

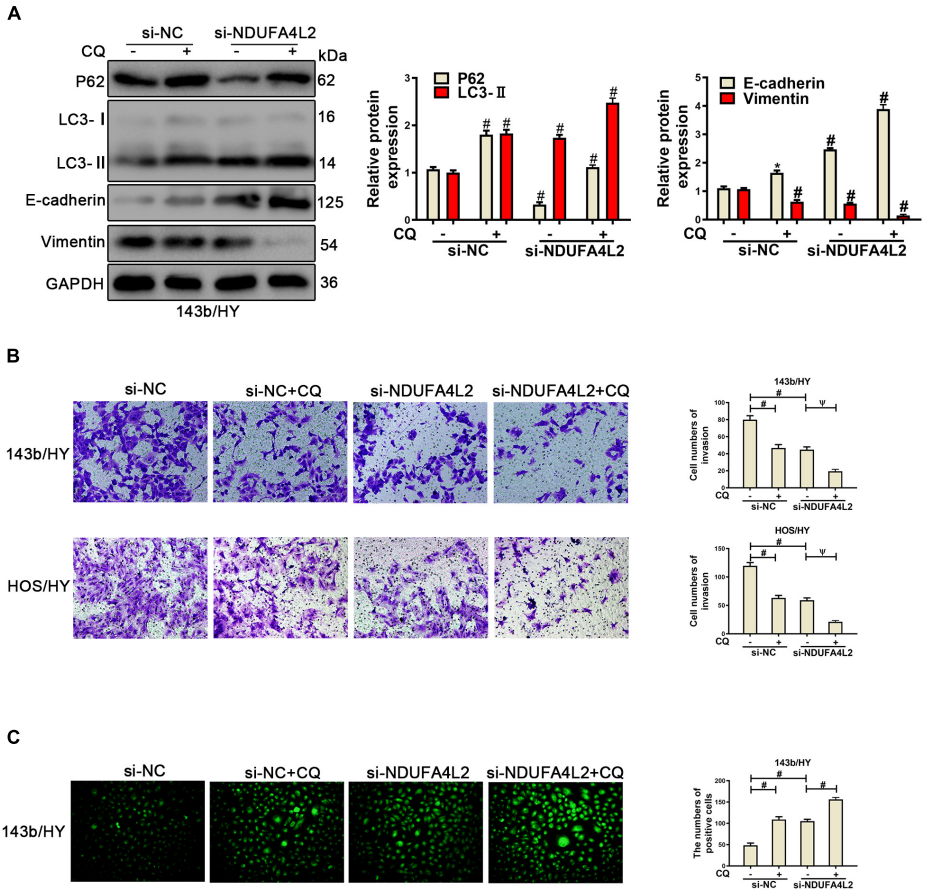

FIGURE 5 | Repression of autophagy could repress the EMT progression of si-NDUFA4L2-transfected 143b and HOS cells. 143b and HOS cells were treated with CQ after transfecting with si-NC or si-NDUFA4L2 in hypoxic environments. **(A)** Western blotting was performed to determine the protein expression of P62, LC3, E-cadherin, Vimentin, and GAPDH in 143b cells. For P62, LC3, E-cadherin, and Vimentin, si-NC vs. si-NC + Rapamycin was # < 0.001, si-NC vs. si-NDUFA4L2 was # < 0.001, si-NDUFA4L2 vs. si-NDUFA4L2 + Rapamycin was # < 0.001. **(B)** Transwell assays were performed to determine the invasion ability of si-NDUFA4L2-transfected 143b and HOS cells after treatment with CQ. **(C)** ROS production was detected by using Reactive Oxygen Detection Kits. HY, hypoxic environment. nsp ≥ 0.05, ψ < 0.01, and # < 0.001 were defined as measures that indicated significant differences among treatment groups. All experiments were performed in triplicate.

(**Supplementary Figure S5F**). These results suggested that NAC could rescue the growth of OS tumors post-knockdown of NDUFA4L2.

DISCUSSION

Osteosarcoma is one of the most common primary malignant bone tumors that occurs in children, teenagers, and young adults. Typical characteristics of OS are pain and swelling in the affected bone in the place of onset. OS patients often wake from sleep with an intensive pain, which is a hallmark of OS (Cai et al., 2019). Chemotherapy is the most efficient supportive therapy for the treatment of OS (Bielack et al., 2002). However, a large number of patients are prone to developing chemoresistance, which might relate to relatively low levels of improvement of respective 5-year survival rates despite advancements in methodology (Ferrari and Serra, 2015). Therefore, there has been an ongoing need for novel methods that can help to better overcome the limitations of chemotherapy. Accordingly, in our study we firstly confirmed that HIF-1α and NDUFA4L2 were overexpressed in OS cells under hypoxic environments. The ROS production and lactate production was increased in hypoxic environments. OCR was decreased in hypoxic environments. We also found that HIF-1α regulated NDUFA4L2 expression through the HRE (Hypoxic reaction element) in the NDUFA4L2 promoter region in OS cell lines cultured in hypoxic environments. HIF-2α did not regulate NDUFA4L2 expression. High levels of expression of NDUFA4L2 were significantly correlated with apoptosis, cell migration, invasion and EMT progression of OS. In 143b and U2OS, we found that knockdown of NDUFA4L2 inhibited OS cell proliferation, invasion, and migration, as well as induced cell apoptosis through the results based upon functional assays. *In vivo*, we found that NDUFA4L2 knockdown could inhibit the growth of OS tumors. These results revealed that NDUFA4L2 induced by HIF-1α improved the survival, metastasis, and EMT progression of OS cells.

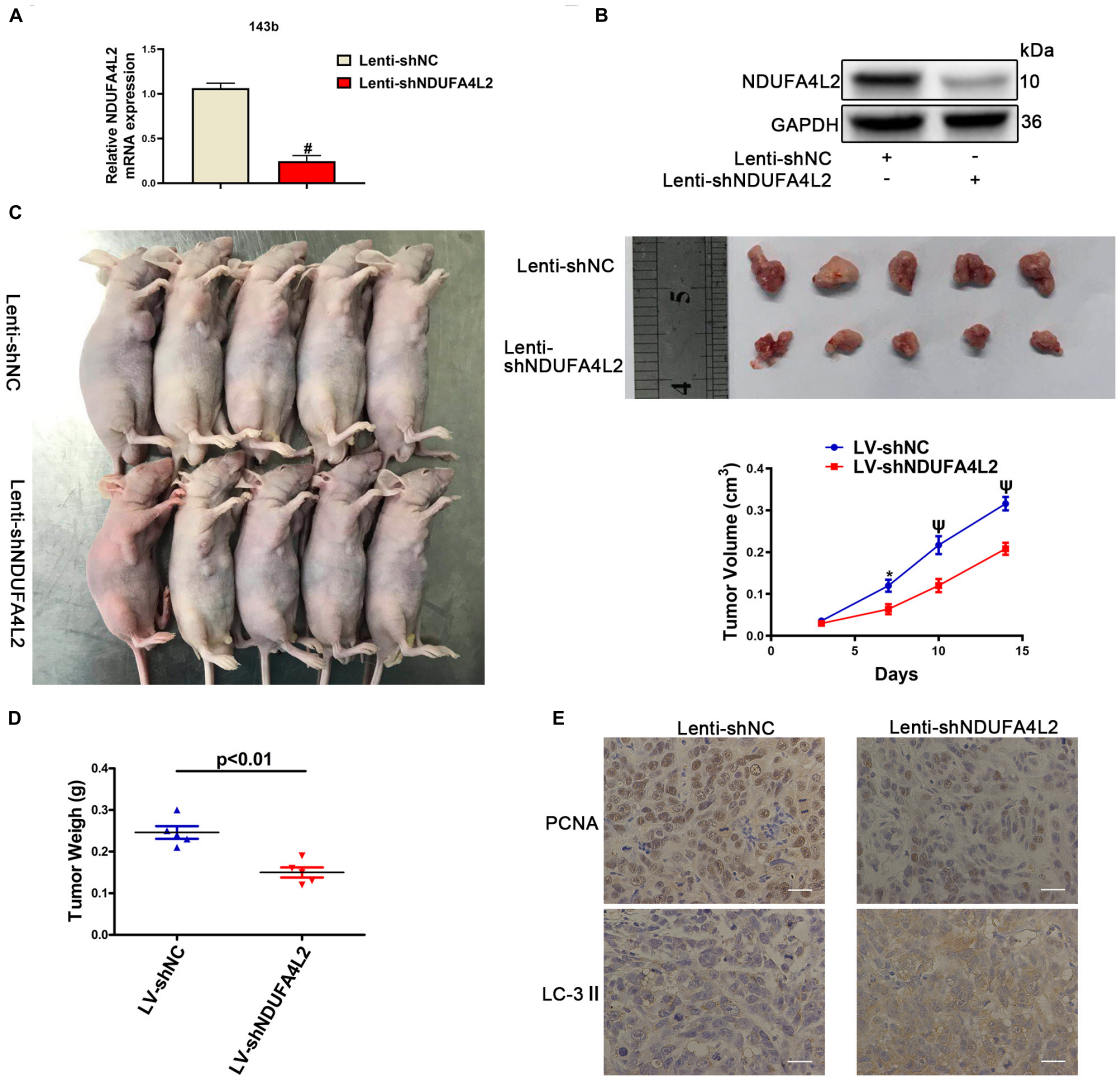

FIGURE 6 | NDUFA4L2 knockdown repressed OS tumor growth *in vivo*. **(A)** The levels of expression of NDUFA4L2 mRNA were detected by using qRT-PCR ($n = 5$). **(B)** NDUFA4L2 protein expression was determined by Western blotting ($n = 5$). **(C)** Results for nude mice carrying tumors from 143b/LV-shNDUFA4L2 and 143b/LV-shNC groups were characterized. Tumor growth curves were assessed weekly ($n = 5$). **(D)** Tumor weight from 143b/LV-shNDUFA4L2 and 143b/LV-shNC groups were characterized ($n = 5$). **(E)** PCNA and LC3 protein expression was determined by using immunohistochemical staining ($n = 5$). nsp ≥ 0.05, $^*p < 0.05$, $\psi < 0.01$, and # < 0.001 were defined as measures that indicated significant differences among treatment groups.

Prior findings have indicated that NDUFA4L2 is upregulated in many kinds of tumors and plays an important role in the hypoxic environments (Tello et al., 2011; Yamamoto and Tsuchiya, 2013; Lai et al., 2016; Wang et al., 2017; Piltti et al., 2018). However, such examinations as related to NDUFA4L2 have yet to be reported upon with respect to OS. In our study, we discovered that NDUFA4L2, a component of the ETC complex I, was upregulated in OS in hypoxic environments. Complex I, which is a key component in the first step of ETC, can transfer electrons from NADPH to a non-covalently bound flavin mononucleotide. In the process of the ubiquinone reduction in complex I and the outer quinone-binding site of the Q cycle in complex III, it has significant ROS production (Raha and Robinson, 2000; Liu et al., 2002; Lv et al., 2017). ROS production mediates homeostasis of redox that correspondingly plays an important role in the survival of cancer cells. In this study, we showed that mitochondrial NDUFA4L2, a component of the ETC complex I subunit, was overexpressed in OS cell lines cultured in hypoxic conditions. OCR was reduced significantly, and the ROS levels was increased in OS cell lines under hypoxic environment. NDUFA4L2 knockdown caused an increased level of ROS and OCR in OS cells under hypoxic environments. These results confirmed that NDUFA4L2 restricted the ETC activity and NDUFA4L2 knockdown increased ROS production and OCR in OS cells through promoting the ETC activity. Based upon our above findings, we inferred that NDUFA4L2 protected OS cell lines from hypoxic environments by facilitating the regulation of redox homeostasis. To test and confirm our hypothesis, the

ROS scavenger NAC was applied to OS cells with the effect of silencing of NDUFA4L2. Interestingly, we discovered that NAC can reverse functions of si-NDUFA4L2 to OS cells. However, NAC did not affect the control cells regardless of whether OS cells were cultured in normal or hypoxic environments (**Supplementary Figure S3A**). From there we evaluated the effects of NAC upon OS cells cultured in normal environments and found that NDUFA4L2 knockdown and NAC did not affect OS cells (**Supplementary Figure S3B**). NDUFA4L2 was downregulated in OS cells cultured in normoxia environments. NDUFA4L2 knockdown did not cause an increase of ROS production and thereby NAC did not affect OS cells cultured in normoxic environments. Overexpression of NDUFA4L2 in normoxia could not activate the EMT progression. *In vivo*, ROS scavenger NAC could promote the growth of OS tumors although NDUFA4L2 was silenced. These results provided support and lent confirmation to our hypothesis that NDUFA4L2 could promote the survival, metastasis, and EMT progression by inhibition of ROS.

It is well-known that autophagy can mediate apoptosis activity. However, the specific mechanism remains unknown. As a double-edged sword, autophagy could either heighten or repress tumor cell proliferation, invasion, and migration, depending upon the conditions of the environment of the cell and stimuli present (St-Pierre et al., 2002). For osteosarcoma, research has contrastingly both observed that autophagy may either promote or inhibit proliferation under the context of different gene regulation (Kenific et al., 2010; Bao et al., 2018; Zhao et al., 2018). Literature has also indicated that autophagy could enhance cellular activity through anti-oxidative stress (Zhang et al., 2018) and that cells tend to increase oxidative stress under hypoxic conditions (Liang et al., 2015). Herein, we found that autophagy related proteins were overexpressed when 143b and U2OS cells were cultured in hypoxic environments. When using small interference to knock down NDUFA4L2, we observed that autophagy flux was upregulated. Treatments with Rapamycin did not enhance the EMT process, invasion, proliferation and migration of OS cells while could reduce ROS levels in hypoxic environments. These results showed that further autophagy could not enhance the tumor phenotype of OS but could compensate for tumor suppression induced by NDUFA4L2 deletion via reducing ROS levels. However, inhibition of autophagy alone could inhibit the tumor phenotype of OS in hypoxic environments and increase the ROS levels. Inhibition of autophagy may cause an increased level of ROS to inhibit the tumor phenotype of OS. Rapamycin promoted OS cell metastasis and EMT progression *in vitro* and CQ enhanced the effect of si-NDUFA4L2 on OS cells when NDUFA4L2 was knocked down. These findings thus revealed that autophagy was able to compensate for the loss of NDUFA4L2 function with respect to OS cell proliferation, invasion, migration and EMT progression. Silencing of NDUFA4L2 was able to induce autophagy to facilitate rescue of proliferation, invasion, migration, and EMT progression.

There is a shortcoming in this study. The further mechanism of NDUFA4L2 in tumor growth has not been fully demonstrated. In future, we hope that our peers will study this issue with us.

CONCLUSION

In conclusion, this study illustrated how the molecular relationship between HIF-1α, NDUFA4L2, oxidative stress, and autophagy mediated the regulation of survival, metastasis and EMT progression of OS cells in hypoxic environments. In hypoxic environments, we found that low oxygen tension induced HIF-1α, such as to promote NDUFA4L2 expression. NDUFA4L2 improved survival, metastasis, and EMT progression of OS cells that survived in hypoxic conditions by facilitating repression of ROS production. When NDUFA4L2 was silenced, a large amount of ROS activated autophagy flux to facilitate maintenance of survival, metastasis, and EMT progression of OS cells. Therefore, the findings in our study helped to elucidate the survival of Osteosarcoma and provided a novel therapeutic target for Osteosarcoma. Further studies are warranted to confirm the clinical significance of NDUFA4L2 and autophagy in Osteosarcoma.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Xinhua Hospital, Shanghai Jiao Tong University School of Medicine.

AUTHOR CONTRIBUTIONS

W-NX, S-DJ, and L-SJ conceived and designed the experiments. W-NX and H-LZ performed the experiments. W-NX and R-ZY acquired and analyzed the data. W-NX drafted the manuscript. S-DJ and L-SJ helped perform the analysis with constructive discussions and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.515051/full#supplementary-material

REFERENCES

Bao, X., Zhao, L., Guan, H., and Li, F. (2018). Inhibition of LCMR1 and ATG12 by demethylation-activated miR-570-3p is involved in the anti-metastasis effects of metformin on human osteosarcoma. *Cell Death Dis.* 9:611.

Bielack, S. S., Kempf-Bielack, B., Delling, G., Exner, G. U., Flege, S., Helmke, K., et al. (2002). Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. *J. Clin. Oncol.* 20, 776–790. doi: 10.1200/jco.20.3.776

Cai, Q., Jin, L., Wang, S., Zhou, D., Wang, J., Tang, Z., et al. (2017). Long non-coding RNA UCA1 promotes gallbladder cancer progression by epigenetically repressing p21 and E-cadherin expression. *Oncotarget* 8, 47957–47968. doi: 10.18632/oncotarget.18204

Cai, Q., Wang, S., Jin, L., Weng, M., Zhou, D., Wang, J., et al. (2019). Long non-coding RNA GBCDRlnc1 induces chemoresistance of gallbladder cancer cells by activating autophagy. *Mol. Cancer* 18:82.

Chen, J. W., Ni, B. B., Zheng, X. F., Li, B., Jiang, S. D., and Jiang, L. S. (2015). Hypoxia facilitates the survival of nucleus pulposus cells in serum deprivation by down-regulating excessive autophagy through restricting ROS generation. *Intern. J. Biochem. Cell Biol.* 59, 1–10. doi: 10.1016/j.biocel.2014.11.009

Ebbesen, P., Eckardt, K. U., Ciampor, F., and Pettersen, E. O. (2004). Linking measured intercellular oxygen concentration to human cell functions. *Acta Oncol.* 43, 598–600. doi: 10.1080/02841860410020220

Ferrari, S., and Serra, M. (2015). An update on chemotherapy for osteosarcoma. *Expert Opin. Pharmacother.* 16, 2727–2736.

Goda, N., Ryan, H. E., Khadivi, B., McNulty, W., Rickert, R. C., and Johnson, R. S. (2003). Hypoxia-inducible factor 1alpha is essential for cell cycle arrest during hypoxia. *Mol. Cell. Biol.* 23, 359–369. doi: 10.1128/mcb.23.1.359-369.2003

Harrison, D. J., Geller, D. S., Gill, J. D., Lewis, V. O., and Gorlick, R. (2018). Current and future therapeutic approaches for osteosarcoma. *Expert Rev. Anticancer Ther.* 18, 39–50.

He, J. N., Zhang, S. D., Qu, Y., Wang, H. L., Tham, C. C., Pang, C. P., et al. (2019). Rapamycin removes damaged mitochondria and protects human trabecular meshwork (TM-1) cells from chronic oxidative stress. *Mol. Neurobiol.* 56, 6586–6593. doi: 10.1007/s12035-019-1559-5

Huang, L. E., Gu, J., Schau, M., and Bunn, H. F. (1998). Regulation of hypoxia-inducible factor 1alpha is mediated by an O_2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc. Natl. Acad. Sci. U.S.A.* 95, 7987–7992. doi: 10.1073/pnas.95.14.7987

Huang, T., Kim, C. K., Alvarez, A. A., Pangeni, R. P., Wan, X., Song, X., et al. (2017). MST4 phosphorylation of ATG4B regulates autophagic activity, tumorigenicity, and radioresistance in glioblastoma. *Cancer Cell* 32, 840–855.e848.

Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gielbert, J., Gaskell, S. J., et al. (2001). Targeting of HIF-alpha to the von hippel-lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. *Science* 292, 468–472. doi: 10.1126/science.1059796

Keith, B., and Simon, M. C. (2007). Hypoxia-inducible factors, stem cells, and cancer. *Cell* 129, 465–472. doi: 10.1016/j.cell.2007.04.019

Kenific, C. M., Thorburn, A., and Debnath, J. (2010). Autophagy and metastasis: another double-edged sword. *Curr. Opin. Cell Biol.* 22, 241–245. doi: 10.1016/j.ceb.2009.10.008

Lai, R. K., Xu, I. M., Chiu, D. K., Tse, A. P., Wei, L. L., Law, C. T., et al. (2016). NDUFA4L2 fine-tunes oxidative stress in hepatocellular carcinoma. *Clin. Cancer Res.* 22, 3105–3117. doi: 10.1158/1078-0432.ccr-15-1987

Li, J., Bai, C., Guo, J., Liang, W., and Long, J. (2017). NDUFA4L2 protects against ischaemia/reperfusion-induced cardiomyocyte apoptosis and mitochondrial dysfunction by inhibiting complex I. *Clin. Exper. Pharmacol. Physiol.* 44, 779–786. doi: 10.1111/1440-1681.12768

Li, P., Li, L., Zhang, C., Cheng, X., Zhang, Y., Guo, Y., et al. (2019). Palmitic acid and beta-hydroxybutyrate induce inflammatory responses in bovine endometrial cells by activating oxidative stress-mediated NF-kappab signaling. *Molecules* 24:2421. doi: 10.3390/molecules24132421

Liang, S., Aiqun, M., Figtree, G., and Ping, Z. (2015). GAPDH-silence preserves H9C2 cells from acute hypoxia and reoxygenation injury. *Int. J. Biol. Macromol.* 81, 375–386. doi: 10.1016/j.ijbiomac.2015.08.028

Lien, W. C., Chen, T. Y., Sheu, S. Y., Lin, T. C., Kang, F. C., Yu, C. H., et al. (2018). 7-hydroxy-staurosporine, UCN-01, induces DNA damage response, and autophagy in human osteosarcoma U2-OS cells. *J. Cell. Biochem.* 119, 4729–4741. doi: 10.1002/jcb.26652

Liu, Y., Fiskum, G., and Schubert, D. (2002). Generation of reactive oxygen species by the mitochondrial electron transport chain. *J. Neurochem.* 80, 780–787. doi: 10.1046/j.0022-3042.2002.00744.x

Lv, Y., Nie, S. L., Zhou, J. M., Liu, F., Hu, Y. B., Jiang, J. R., et al. (2017). Overexpression of NDUFA4L2 is associated with poor prognosis in patients with colorectal cancer. *ANZ J. Surg.* 87, E251–E255.

Maxwell, P. H., Wiesener, M. S., Chang, G. W., Clifford, S. C., Vaux, E. C., Cockman, M. E., et al. (1999). The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* 399, 271–275. doi: 10.1038/20459

Meng, L., Yang, X., Xie, X., and Wang, M. (2019). Mitochondrial NDUFA4L2 protein promotes the vitality of lung cancer cells by repressing oxidative stress. *Thorac. Cancer* 10, 676–685. doi: 10.1111/1759-7714.12984

Mowers, E. E., Sharifi, M. N., and Macleod, K. F. (2017). Autophagy in cancer metastasis. *Oncogene* 36, 1619–1630.

Muz, B., de la Puente, P., Azab, F., and Azab, A. K. (2015). The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. *Hypoxia* 3, 83–92. doi: 10.2147/hp.s93413

Piltti, J., Bygdell, J., Qu, C., and Lammi, M. J. (2018). Effects of long-term low oxygen tension in human chondrosarcoma cells. *J. Cell. Biochem.* 119, 2320–2332. doi: 10.1002/jcb.26394

Raha, S., and Robinson, B. H. (2000). Mitochondria, oxygen free radicals, disease and ageing. *Trends Biochem. Sci.* 25, 502–508.

St-Pierre, J., Buckingham, J. A., Roebuck, S. J., and Brand, M. D. (2002). Topology of superoxide production from different sites in the mitochondrial electron transport chain. *J. Biol. Chem.* 277, 44784–44790. doi: 10.1074/jbc.m207217200

Tello, D., Balsa, E., Acosta-Iborra, B., Fuertes-Yebra, E., Elorza, A., Ordóñez, Á, et al. (2011). Induction of the mitochondrial NDUFA4L2 protein by HIF-1alpha decreases oxygen consumption by inhibiting complex I activity. *Cell Metab.* 14, 768–779. doi: 10.1016/j.cmet.2011.10.008

Torre, L. A., Bray, F., Siegel, R. L., Ferlay, J., Lortet-Tieulent, J., and Jemal, A. (2015). Global cancer statistics, 2012. *CA Cancer J. Clin.* 65, 87–108. doi: 10.3322/caac.21262

Umezawa, K., Kojima, I., Simizu, S., Lin, Y., Fukatsu, H., Koide, N., et al. (2018). Therapeutic activity of plant-derived alkaloid conophylline on metabolic syndrome and neurodegenerative disease models. *Hum. Cell* 31, 95–101. doi: 10.1007/s13577-017-0196-4

Wang, L., Peng, Z., Wang, K., Qi, Y., Yang, Y., Zhang, Y., et al. (2017). NDUFA4L2 is associated with clear cell renal cell carcinoma malignancy and is regulated by ELK1. *PeerJ* 5:e4065. doi: 10.7717/peerj.4065

Weckman, A., Rotondo, F., Di Ieva, A., Syro, L. V., Butz, H., Cusimano, M. D., et al. (2015). Autophagy in endocrine tumors. *Endocr. Relat. Cancer* 22, R205–R218.

Xie, L., Pi, X., Mishra, A., Fong, G., Peng, J., and Patterson, C. (2012). PHD3-dependent hydroxylation of HCLK2 promotes the DNA damage response. *J. Clin. Invest.* 122, 2827–2836. doi: 10.1172/jci62374

Yamamoto, N., and Tsuchiya, H. (2013). Chemotherapy for osteosarcoma - where does it come from? What is it? Where is it going? *Expert Opin. Pharmacother.* 14, 2183–2193. doi: 10.1517/14656566.2013.827171

Zhang, F., Yan, T., Guo, W., Sun, K., Wang, S., Bao, X., et al. (2018). Novel oncogene COPS3 interacts with Beclin1 and Raf-1 to regulate metastasis of osteosarcoma through autophagy. *J. Exper. Clin. Cancer Res.* 37:135.

Zhao, G. S., Gao, Z. R., Zhang, Q., Tang, X. F., Lv, Y. F., Zhang, Z. S., et al. (2018). TSSC3 promotes autophagy via inactivating the Src-mediated PI3K/Akt/mTOR pathway to suppress tumorigenesis and metastasis in osteosarcoma, and predicts a favorable prognosis. *J. Exper. Clin. Cancer Res.* 37:188.

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