Review Article

The role of the bone microenvironment in skeletal metastasis

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ABSTRACT

The bone microenvironment provides a fertile soil for cancer cells. It is therefore not surprising that the skeleton is a frequent site of cancer metastasis. It is believed that reciprocal interactions between tumour and bone cells, known as the "vicious cycle of bone metastasis" support the establishment and orchestrate the expansion of malignant cancers in bone. While the full range of molecular mechanisms of cancer metastasis to bone remain to be elucidated, recent research has deepened our understanding of the cell-mediated processes that may be involved in cancer cell survival and growth in bone. This review aims to address the importance of the bone microenvironment in skeletal cancer metastasis and discusses potential therapeutic implications of novel insights.

1. Introduction

Bone metastases are a major cause of cancer-related pain and can result in pathological fractures, paralysis and life-threatening hypercalcaemia. Less than 20% of patients survive for five years after the discovery of bone metastasis [1–4]. In other types of cancers, such as liver and lung malignancies, the incidence of bone metastasis has increased in recent years, possibly due to the effect of improved treatment regimens on life expectancy [5,6].

Metastasis of tumour cells to bone depends on a complex cascade of events which includes the detachment of individual cancer cells from the primary tumour site; invasion into the vasculature; migration and adherence to distant capillaries within the bone; extravasation and initial survival within the new environment; proliferation to micrometastases; recruitment of blood supply to the tumour for further expansion; and invasion beyond the adjacent tissues [3,4,7]. The ability of cancer cells to survive and expand in the bone marrow cavity has long been based on the "seed and soil" theory: In 1889, Sir James Paget proposed that bone acts as a fertile environment ('soil') for cancer cell ('seed') colonization and growth [8]. Many years later, Mundy and colleagues greatly broadened our understanding of the mechanisms that govern the growth of bone metastases by developing a concept

widely known as the "vicious cycle" [7,9–11]. This theory elegantly explains how cancer metastases, once established in bone, modify their immediate environment to support their own survival and growth. Thus, tumour-derived factors such as parathyroid hormone-related protein (PTHrP) up-regulate the expression of Receptor Activator of Nuclear Factor KB Ligand (RANKL) by cells of the osteoblast lineage (i.e., osteoblast precursors, osteoblasts and osteocytes). RANKL then binds to the Receptor Activator of Nuclear Factor KB (RANK) on osteoclasts and osteoclast precursors to increase osteoclast recruitment and formation, and to activate bone resorption. Accelerated bone resorption then triggers the release of growth factors embedded in the bone matrix, which in turn act on cancer cells to promote their further growth [7,10,12] (Fig. 1). This model has been extremely useful in elucidating some of the mechanisms that support and maintain established cancer metastases in bone. It is, however, less clear how individual cancer cells survive and proliferate within the bone environment at the very early stages of colonisation, i.e., before reaching a critical mass that allows them to manipulate resident bone cells in a significant way. We would therefore predict that additional mechanisms are at work at the early stages of bone metastases that involve more direct signalling pathways than those described by the classical vicious pathway.

Numerous animal studies have demonstrated beyond doubt that effective inhibition of osteoclastogenesis or osteoclast function significantly reduces metastatic tumour growth in bone [13–20]. Likewise, clinical trials in patients with non-metastatic or metastatic cancers established that treatment with "anti-resorptive" agents such as bisphosphonates or the anti-RANKL antibody,

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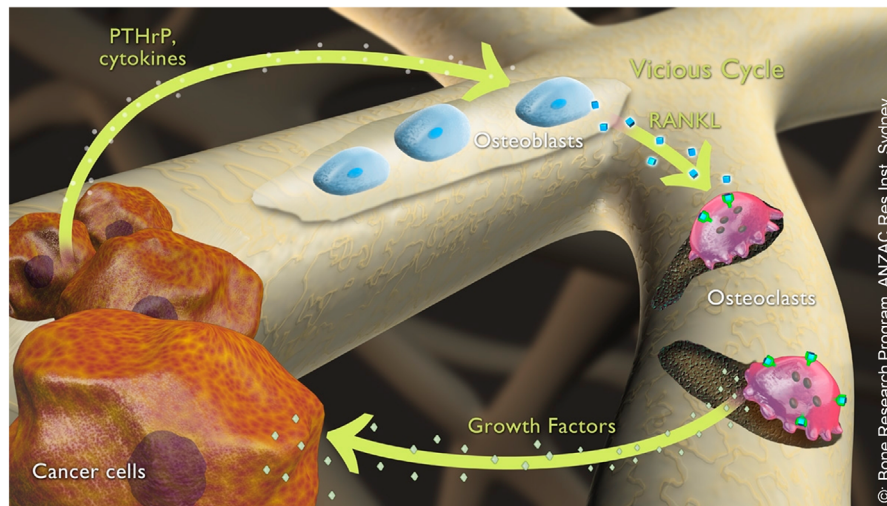
Fig. 1. Schematic representation of the 'vicious cycle'. Up-regulation of RANKL in bone cells and subsequent osteoclast activation is driven primarily by tumour-derived factors such as PTHrP and IL-6. Accelerated bone resorption then triggers the release of growth factors from the degraded bone matrix, which in turn promote further tumour growth.

denosumab, resulted in significant reductions in the incidence, progress or complications of bone metastases [21–23]. Despite these significant developments, complications of bone metastases still occur in up to 50% of patients even whilst receiving anti-resorptive therapy [1,4], indicating that there are still significant unmet needs in the prevention and treatment of metastatic bone disease.

2. Types of bone metastasis

Bone metastases have generally been characterized as osteo-lytic or osteoblastic based on their radiographic appearance [1]. Osteolytic lesions are caused by increased osteoclast activity accompanied by a concomitant absolute or relative decrease in osteoblast number or activity. This results in net bone resorption [7,24] with little or no associated bone repair. In contrast, osteoblastic lesions are characterized by abnormal bone forma-tion around tumour cell foci, but this typically also co-exists with increased osteoclast activity. Thus, both types of cancer metas-tasis to bone are characterised by significantly accelerated bone resorption with the radiographic appearance depending on the concurrent levels of bone formation. These tumour-induced changes in bone metabolism can clinically be identified and monitored through the measurement of bone turnover markers, which correlate with both tumour burden and therapy-induced reductions in skeletal related events [1,25–35]. Thus, the classi-fication of metastatic bone lesions into osteolytic and osteoblastic represent no less than the two extremes of a continuum in which the normal bone remodelling process becomes dysfunctional. Furthermore, patients can present with both osteolytic and osteoblastic lesions, and in fact, many bone metastases are mixed in nature, containing both lytic and blastic elements [12]. For example, breast cancer predominantly causes osteolytic metas-tases but at least 20% of patients present with mixed osteolytic-osteosclerotic lesions [2]. Conversely, prostate cancer presents mostly with osteoblastic lesions although a concurrent increase in bone resorption invariably occurs [2,4,36]. In patients with advanced bone metastases, high circulating levels of bone resorp-tion markers, such as the aminoterminal telopeptide of type I collagen (NTX), were seen regardless of whether the lesions were radiographically lytic, blastic or "mixed" [30,37,38]. This indicates that all types of bone metastases contain an element of osteoclast

activation, and this has been confirmed histologically. The role of osteoclasts in the spectrum of metastatic bone lesions is also supported by the fact that anti-resorptive therapy effectively reduces skeletal related events independent of whether there is predominantly lytic or blastic metastatic bone disease [23,39,40].

Within the bone microenvironment, the establishment of a tumour thus results in a disruption of the normally well-coordinated coupling of osteoblast and osteoclast functions. The resulting abnormal and accelerated bone remodelling then offers a fertile soil for further tumour expansion. Therefore, when it comes to the understanding of the mechanisms that enable cancers to grow in bone, the role of the bone microenvironment and its manipulation by the cancer cannot be underestimated.

3. The bone microenvironment

The term 'bone microenvironment' attempts to describe a complex structural and biological system which contains both haematopoietic and mesenchymal cells of multiple lineages, a sinusoidal blood supply, the bone marrow stroma and the bone extracellular matrix. In the context of skeletal cancer metastases, the bone matrix serves as a rich source of growth factors, while a number of different cells types inside, or recruited to the bone marrow cavity function to orchestrate the bone-tumour interac-tions. The cells within the bone microenvironment include resident bone cells (osteoclasts, osteoblasts and osteocytes) as well as various other cell types such as myeloid and immune cells, platelets, bone marrow endothelial and haematopoietic cells and bone marrow-derived mesenchymal stem cells, all of which may engage with the metastatic process to varying degrees.

3.1. Role of the bone matrix

Over the past 30 years it has become apparent that the bone matrix is extremely rich in growth factors. Many of these, including TGFβ, IGFs, FGFs, PDGF and BMPs not only promote the growth of metastatic cancer cells in bone, but also increase the production and release of cytokines and other bone resorbing factors from tumour cells [1,41]. Growth factors released by the bone matrix are able to change the phenotype of tumour cells to cause more aggressive metastatic lesions [3,7]. To again use Paget's analogy: The bone 'soil' is 'fertilized' by matrix-derived growth factors to

facilitate the growth of the cancer 'seed' [1,3,7]. These factors can be released into the bone microenvironment during bone remodelling [7,12,42,43]. Physical properties of the bone matrix, its structure and local changes associated with remodelling activity, including hypoxia, acidosis, and high extracellular calcium concentrations, create an environment favourable for tumour cells and their growth [12,44,45]. Finally, the bone matrix contains numerous non-collagenous proteins (e.g., osteopontin, vitronectin) which are able to interact directly with adhesion molecules on cancer cells, commonly via RGD sequences, and thus alter cancer cell behaviour.

3.2. Bone remodelling and bone cells

Bone is continuously moulded, shaped and repaired through the actions of different bone cells [46]. During development and growth, the skeleton is built up to achieve its shape and size by the removal of bone from some sites and deposition (synthesis) of bone at other sites; this process is called bone **modelling** [47,48]. Once the skeleton has reached its mature size and structure, another life-long process termed **remodelling** commences, resulting in the continuous replacement of 'old' bone by newly formed tissue in the same location. Bone remodelling generally occurs at a micro-scale throughout the skeleton, carried out by the coordinated activity of juxtaposed osteoblasts and osteoclasts, an entity also known as a 'basic multicellular unit (BMU)' [47].

During bone remodelling, osteoblasts and osteoclasts are in intimate contact with the bone marrow, from which their precursors are derived. The differentiation of osteoclasts from the macrophage/monocyte lineage and their subsequent activation initially result in bone resorption, either on the bone surface or by tunnelling into the bone matrix [49]. A reversal phase then follows and a cement line is deposited. During the final stages of bone remodelling, osteoblasts (cells of mesenchymal origin) lay down new bone matrix which subsequently becomes mineralised [42]. Osteocytes are cells derived from osteoblasts [50] which become embedded in the newly formed bone matrix. Bone surfaces then remain in a resting state of variable duration until the next remodelling cycle begins [48].

During adult life, balanced bone remodelling is the major process by which healthy bone structure and function are maintained. In the young adult, several million bone remodelling units work their way through the skeleton at any one time, resorbing old bone and replacing it with an equal amount of new bone, such that total bone mass remains unchanged. During aging (which for the skeleton starts around 40 years of age), bone remodelling becomes increasingly imbalanced, and a shift in favour of net bone resorption occurs [46,48].

The bone remodelling process is under the control of osteoblasts which integrate the signalling input from systemic hormones, locally acting growth factors and cytokines, and mechanical stress. Formation of osteoclasts occurs through a sequence of events that includes proliferation, differentiation, fusion and activation [12,43,51]. These events are regulated via the RANKL/ RANK/OPG signalling system (see below) [49].

3.3. The RANKL/RANK/OPG system

The receptor activator of NF Kappa B (RANK) ligand (RANKL) belongs to the TNF super family and is expressed by several cell types within the bone environment, including osteoblasts, other cells of the osteoblast lineage and T-cells. RANKL has been identified as the key signal in the regulation of osteoclastogenesis and bone resorption [52–55]. Specifically, RANKL binds to its receptor, RANK, a transmembrane signalling receptor expressed by haematopoietic osteoclast precursor cells [52] and induces

their differentiation into functional, multinucleated osteoclasts. RANKL also promotes osteoclast activation and survival [1,7]. Gene knockout experiments further reveal the physiological importance of RANK and RANKL and their interactions. Thus, mice deficient in either RANK or RANKL are phenotypically identical, each presenting with profound osteopetrosis and an absence of osteoclasts. These phenotypes clearly demonstrate the essential role of this receptor-ligand pair in bone modelling and remodelling [56,57].

The RANK–RANKL system is further regulated through osteoprotegerin (OPG), which acts as a decoy receptor to RANKL, preventing RANKL from binding to RANK. Interestingly, OPG is expressed also by osteoblasts as a secreted soluble protein. Through its ability to block osteoclast differentiation and activation (i.e., bone resorption), OPG becomes an important counter-regulator of bone metabolism [54,58]. When administered systemically, OPG has been shown to inhibit both physiologic and pathologic bone resorption in various animal models, including those of metastatic bone disease [15,20,58–61]. However, it is the ratio of RANKL to OPG rather than the absolute levels of either that determines the level of osteoclastogenesis in vivo [54,58].

Numerous osteotropic hormones and cytokines are able to influence the expression levels of both OPG and RANKL [62,63]. Systemic factors such as parathyroid hormone (PTH), interleukins and tumour necrosis factor α (TNF-α) increase the osteoblastic expression of RANKL relative to that of OPG, thereby promoting osteoclast activity [7]. Conversely, treatment with OPG has been shown to effectively inhibit bone resorption in humans [64,65]. Importantly, the same RANKL/RANK/OPG pathways are operational in humans and rodents.

4. The 'vicious cycle' of metastatic tumour growth in bone

The concept of a 'vicious cycle' supporting and maintaining metastatic tumour growth in bone was first introduced by Mundy and Guise in 1997 [66]. The model successfully explains how bone and cancer cells interact in a feed-forward loop to allow and perpetuate cancer cell growth within the bone microenvironment. In its essence, the model describes how tumour cells communicate with osteoblasts, which ultimately leads to osteoclast activation and accelerated bone resorption. This not only makes room for the cancer to grow, but also triggers the release of growth factors embedded into the degraded bone matrix. These growth factors then promote further tumour growth, resulting in the production of more pro-resorptive signals by the cancer (Fig. 1).

4.1. The vicious cycle

Factors secreted by tumour cells play a critical role in cancer bone metastasis. Thus, it has been well established that breast or prostate cancer cells are able to produce and release signalling molecules that have the potential to modulate normal bone remodelling. These include parathyroid hormone-related protein (PTHrP), interleukins 6 (IL-6), 8 (IL-8) and 11 (IL-11), as well as vascular endothelial growth factor (VEGF) [3,12,67–71]. Among these factors, the most extensively studied is PTHrP, which initially was identified as the causal factor in humoral hypercalcemia of malignancy (HMM) [69,72–75]. PTHrP shares a common receptor with parathyroid hormone (PTH) [76], and although the protein sequence of PTHrP is different from PTH, there is an approximately 70% sequence homology between the two hormones across the first 13 amino acids at the N-terminus, resulting in similar biological activities [77]. Tumour-derived PTHrP can indirectly activate osteoclastogenesis via osteoblasts by stimulating osteoblasts and stromal cells to increase

RANKL and suppress OPG expression [78–80]. Mouse models of bone metastasis provide solid evidence that PTHrP plays a critical role in breast cancer bone metastasis [81] (Fig. 2). Blocking PTHrP with neutralizing antibodies reduced osteolytic lesions in the MDA-MB-231 mouse model [82]. Interestingly, however, patients with PTHrP-positive primary breast tumours were found to be at *lower* risk of developing bone metastasis and to have a better overall prognosis than patients with tumours that express no or little PTHrP [71,83]. These intriguing results suggest that increased expression of PTHrP by breast cancer is correlated with a less invasive phenotype, indicating that PTHrP may have other effects on tumour cell behaviour which are independent of their local effects on bone following bone metastasis [39,71].

Other factors able to stimulate osteoclast formation and subsequently osteolytic activity include interleukins 6, 8, 11, TNF-α, M-CSF and endothelial growth factor (VEGF) [12,84]. IL-6, IL-11 and VEGF all increase osteoclast formation and activity via the RANK ligand pathway, while macrophage colony stimulating factor (M-CSF) and IL-8 act directly to stimulate human osteoclast formation [85–87].

Up-regulation of RANKL in osteoblasts and other cells of the osteoblast lineage leads to osteoclast activation and increased bone resorption. This has two effects: First, it removes existing bone, thus eliminating an important barrier to further tumour expansion. Second, and as mentioned above, the bone microenvironment is a fertile soil for metastatic tumour cells, given its abundance in growth factors and cytokines. Breakdown of the bone matrix during bone resorption results in the release of potent growth factors, which in turn stimulate tumour growth.

Among the factors, TGF-β is of particular importance as it has been shown to increase the production of PTHrP in breast cancer cells [70,81]. Preclinical studies indicate that blocking TGF-β signalling may have clinical benefits in patients with bone metastases [88–91]. Likewise, various other studies suggest that targeting bone-derived IGF-I, PDGF and BMP and their associated signalling pathways may offer potential therapeutic value in the treatment of bone metastasis [92–96].

It is clear that disruption of the vicious cycle at any level should result in an inhibition of metastatic tumour growth, and in contrast, increased bone resorption is likely to enhance cancer growth in bone [7,24].

4.2. Anti-resorptive treatments reduce metastatic tumour growth in bone

Xenograft models of malignant bone disease have provided clear evidence that inhibiting osteoclast activity and hence bone resorption strongly affects the ability of cancer cells to grow within the bone environment [13–20]. Currently, two main classes of anti-resorptive treatments are available: Bisphosphonates and the anti-RANKL antibody, denosumab. While initially developed for the management of osteoporosis, these agents were subsequently found to also reduce skeletal-related events in patients with metastatic bone disease. Bisphosphonates have successfully been used in the treatment of malignant hypercalcemia and skeletal metastasis in breast and prostate cancers [18,97]. Both animal and human studies have demonstrated that bisphosphonates not only
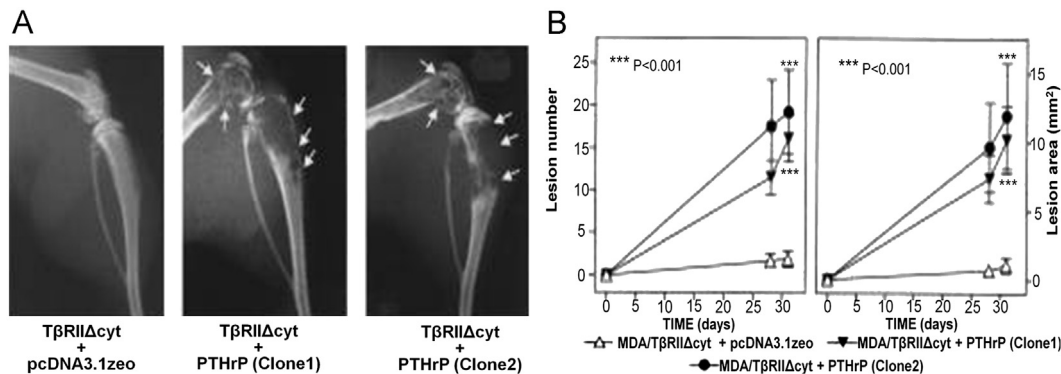

Fig. 2. Overexpressing PTHrP in MDA-MB-231 cells accelerates bone metastases. MDA/TβRIIΔcyt cells were created in MDA-MB-231 cells that expressed the dominant–negative of the TGF-β type II receptor rendered the human breast cancer cell line MDA-MB-231 unresponsive to TGF-β. MDA-MB-231 and MDA/TβRIIΔcyt cell clones that overexpress PTHrP (TβRIIΔcyt+PTHrP; two clones) or the empty vector (TβRIIΔcyt+pcDNA3.1zeo) were used. (A) Representative radiographs of hindlimbs from mice bearing two different TβRIIΔcyt+PTHrP clones or TβRIIΔcyt+pcDNA3.1zeo control 31 days after tumour inoculation. Osteolytic lesions are indicated by the arrows. (B) Osteolytic lesion number and area on radiographs as measured by computerized image analysis of forelimbs and hindlimbs. Respective tumour cells were inoculated on day 0. Values represent the mean ± SEM ($n=5$) per group.
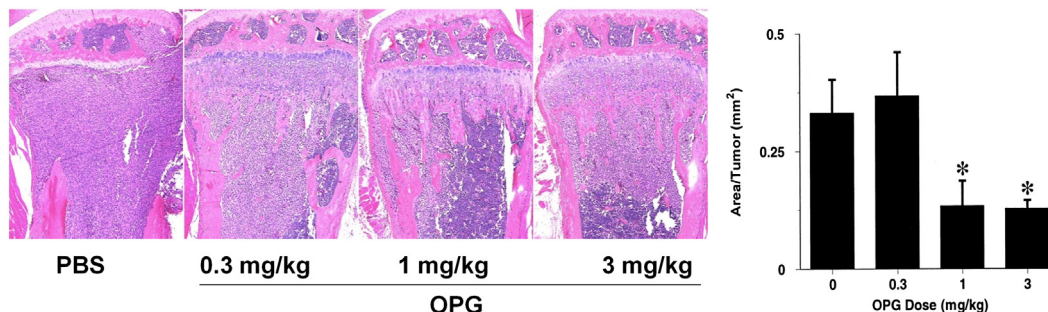From Ref. [81] with permission from the Publisher.

Fig. 3. Effect of OPG treatment on histomorphometric indices of skeletal Colon-26 tumour burden. OPG treatment (1 mg/kg and 3 mg/kg) significantly reduced the average tumour area at each dose. *, Significantly different from 0 mg/kg OPG. Data represent the means ± SE ($n=10$ mice/group).
From Ref. [13] with permission from the Publisher.

reduce osteolysis and bone pain associated with cancer metastasis but also decrease skeletal tumour burden. One area of contention is whether these effects are solely due to inhibiting bone resorption, and thus through altering the "vicious cycle", or whether any of these treatments have direct cytotoxic effects on cancer cells. As discussed below, bisphosphonates and OPG (or indeed denosumab) inhibit bone resorption by different mechanisms, and may therefore possess different anti-tumour potential. Earlier studies with OPG had already demonstrated a functional role for RANKL-induced osteoclastogenesis in humoral hypercalcemia of malignancy and solid malignancies of bone [13,58] (Fig. 3). Beneficial effects of anti-resorptive treatments on malignant bone lesions have been reported across the continuum of lytic to sclerotic lesions for diverse tumour types including prostate, breast, lung and other epithelial tumours [12,98–102].

4.2.1. Bisphosphonates

Bisphosphonates are analogues of the naturally occurring compound pyrophosphate (P–O–P) in which the oxygen in 'P–O–P' has been replaced by a carbon atom, resulting in a 'P–C–P' structure [103–105]. Bisphosphonates bind preferentially to bone minerals at sites of active bone resorption, where they are taken up by resorbing osteoclasts. Once within the cell, the newer nitrogen-containing bisphosphonates inhibit farnesyl pryrophosphate synthase and prevent protein prenylation, which interferes with normal cell metabolism and induces a profound decline in osteoclast-mediated bone resorption [104,106,107]. Some bisphosphonates (e.g., ibandronate) have also been shown to exert direct anti-tumour effects in vitro, *albeit* at micromolar, i.e., exceedingly high concentrations [108,109]. However, since sequestered bisphosphonate can be released from the bone matrix during bone resorption, some cancer cells may indeed be exposed to relatively high BP concentrations in vivo. For example, alendronate is believed to reach concentrations of greater than 100 μM within the sealed zone of an osteoclast during

bone resorption [110], potentially producing transient high levels that may affect cancer cells immediately adjacent to osteoclasts. Whether bisphosphonates do posses direct anti-tumour effects in vivo remains an open question.

Alternatively, it has been suggested that the anti-resorptive activity of bisphosphonates mediate an indirect anti-tumour effect via the vicious cycle. In a study comparing ibandronate and OPG in a breast cancer bone metastasis in mice, we found similar inhibition of tumour growth with both agents, suggesting that their common anti-resorptive actions are dominant in their anti-tumour effects [15] (Fig. 4).

For more than two decades, bisphosphonates have been used as highly effective therapies for the treatment of skeletal malignancies and the prevention of secondary complications [97,111–113]. Anti-resorptive treatment of patients with early, non-metastatic breast cancer with clodronate has been reported to have beneficial effects on both the development of bone metastases and patient survival [38,114–118]. Furthermore, oral clodronate, when given as adjuvant therapy over 5 years, was shown to significantly reduce the rate of bone metastasis in women with breast cancer receiving standard treatment [115,119]. Although one clinical trial reported that clodronate had no effect on metastases and even a negative effect on survival [118,120], bisphosphonates have been widely adopted in clinical practice [121,122] based on further positive outcomes from studies with zoledronic acid [123–134] and ibandronate [135,136] suggesting that bisphosphonates limit the progression of breast or prostate cancer in bone and other tissues [104,114].

Recent results from clinical trials (e.g., AZURE, ABCSG-12, ZO-FAST) suggest that zoledronic acid may have more pronounced effects on the prevention and treatment of breast cancer patients within a low-estrogen environment, i.e., in postmenopausal women [126–134]. This effect is likely due to increases in local and systemic bone resorption in the setting of sex hormone deficiency, as high bone turnover is potentially associated with
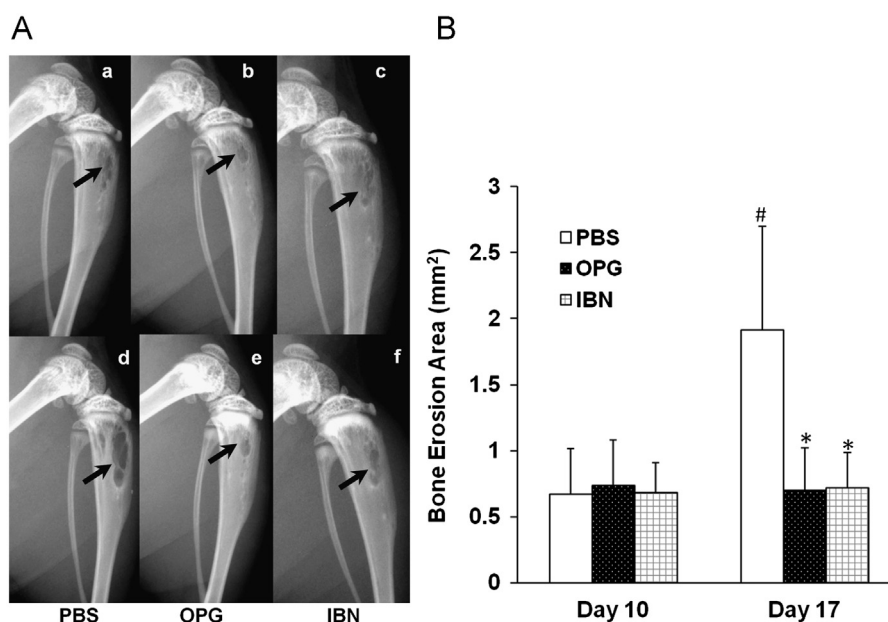

Fig. 4. Osteoprotegerin and ibandronate treatment completely inhibits the enlargement of osteolytic bone lesions. (A) Representative radiographs of osteolytic lesions in tibiae of nude mice before treatment (Day 10; a–c) or after treatment (Day 17; d–f) with vehicle (PBS) osteoprotegerin (OPG) or ibandronate (IBN). At day 10, small but distinct osteolytic lesions (arrows) are detected in the tibiae (a–c). The size of these osteolytic lesions in untreated bones is markedly increased 7 days later, at day 17 (e). In contrast, in all treated bones, increase in size of these lytic lesions is inhibited (e–f). (B) Effects of osteoprotegerin (OPG) and ibandronate (IBN) treatment on the progression of established osteolytic bone lesions. Data are mean $\pm$ SD and $n = 10$ in each group. *significantly different from vehicle-treated group at Day 17 ($p < 0.01$), #different to Day 10 ($p < 0.01$).
From Ref. [15] with permission from the Publisher.

cancer metastasis (see 4.3 below). In addition to its effects on bone resorption, zoledronic acid may or may not have direct anticancer activity. This, however, is a complex question requiring further research. Indeed, clinical data [130,132,133,137] suggest that both hormone suppression and a reduction in bone turnover may be required to achieve sufficient suppression of dormant micrometastases in patients with early-stage breast cancer in the menopausal women.

4.2.2. Anti-RANKL treatments

As a decoy receptor for RANKL, OPG has potent anti-resorptive effects without direct cytotoxic actions [13,20,98]. Binding of RANKL to its cognate receptor RANK on the surface of osteoclast precursor cells is essential for osteoclast differentiation. By sequestering RANKL, OPG inhibits osteoclastogenesis and thus bone resorption in vitro and in vivo [55,56,58,138,139]. In clinical trials, recombinant OPG constructs and anti-RANKL antibodies (denosumab) were demonstrated to reduce bone resorption effectively in patients with multiple myeloma or bone metastasis from breast cancer[140,141]. In prostate cancer, treatment with denosumab has been reported to delay the appearance of bone metastases [142]. Further studies demonstrated that denosumab significantly decreased skeletal complications and reduced bone pain [23]. Interestingly, a recent paper highlights the bone-independent role of RANKL in mammary gland development in mice, where it appears to mediate progesterone-induced proliferation. This data implies that denosumab may be effective in directly targeting subtypes of breast and prostate cancers that express RANKL [143]. Both bisphosphonates and denosumab have been shown to be effective in reducing cancer-induced bone pain [34,144,145]. It is likely that these effects, too, are related to the strong anti-resorptive activity of these agents.

4.3. High bone turnover is causally associated with cancer metastasis

While many studies demonstrated that blocking bone resorption inhibits or even prevents the establishment and growth of tumour cells in the bone environment, investigations into the effects of accelerated bone resorption on tumour growth are scarce. One study reported enhanced cancer cell growth in bone following ovariectomy [146]. Other groups found faster tumour growth during treatment with G-CSF [147] or 17-allylamino-17-demethoxygeldanamycin (17-AAG) [148], although whether the effects on tumour growth were caused by an increase in bone resorption or mediated via other effects related to the bone marrow environment remained uncertain.

Clinical studies have reported that cancer patients with high levels of bone resorption at baseline or on treatment are at higher risk for adverse clinical outcomes, such as SREs or tumour progression [26–32,37,149–154]. In this context, it is interesting to note that calcium and/or vitamin D deficiency have their own and well established effects on bone turnover. Both conditions, which are common in the general but particularly in the older population, are often associated with hyperparathyroidism and, consequently, accelerated bone turnover. Of note, vitamin D deficiency has been identified in epidemiological studies as a risk factor for breast and prostate cancer progression [31,32,155–157]. However, very little was known about the effects of calcium deficiency on skeletal cancer progression.

Over the past years, we have therefore investigated the complex relationship between tumour growth in bone, vitamin D or calcium deficiency, and bone turnover. To this aim, we first created a model of calcium deficiency by restricting dietary calcium intake in young growing nude mice [158]. Within 3 days,

Table 1
Levels of serum calcium, parathyroid hormone and bone markers in mice maintained on a normal or a low calcium diet.

Serum assay	Normal-Ca	Low-Ca
Day 0 ($n=5$)		
Calcium (mmol/l)	2.22 ± 0.04	2.11 ± 0.02*
PTH (pg/ml)	31.01 ± 4.50	63.13 ± 16.99*
mTRAP5b (b U/l)	8.66 ± 0.61	11.78 ± 1.17*
Osteocalcin (ng/ml)	179.24 ± 13.92	267.81 ± 16.91*

PTH, serum intact parathyroid hormone; mTRAP5b, serum mouse tartrate-resistant acid phosphatase 5b; OPG, osteoprotegerin.
Data are expressed as mean $\pm$ SE.
$p < 0.05$ v.s. Normal-Ca.
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Table 2
Bone histomorphometry of the tibiae of mice maintained on a normal or low calcium diet.

	Normal-Ca	Low-Ca
BV/TV (%)	11.67 ± 0.47	9.03 ± 0.62*
No/BS	7.19 ± 0.23	9.33 ± 0.35*
Oc.S/BS (%)	40.41 ± 1.46	52.68 ± 1.53*
Ob.S/BS (%)	24.60 ± 1.37	38.03 ± 2.31*

BV/TV: Bone volume % tissue volume.
N.Oc/BS: Osteoclast number per mm bone surface.
Oc.S/BS: Osteoclast surface % bone surface.
Ob.S/BS: Osteoblast surface % bone surface.
Data are expressed as mean $\pm$ SE, * $p < 0.05$ v.s. Normal-Ca, $n=5$/group.
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these mice develop secondary hyperparathyroidism (Table 1) and accelerated bone turnover, resulting in significant bone loss (Table 2). Using this model, we were able to demonstrate that calcium deficiency in mice significantly stimulated the growth of two human breast cancer cell lines (MDA-MB-231 and MCF-7) implanted intra-tibially into bone (Fig. 5) [158,159]. Since vitamin D is a major regulator of calcium homeostasis, we proceeded to develop a rodent model of vitamin D deficiency [60]. After weaning, 3-week-old nude mice were provided with either normal chow (1000 IU/kg cholecalciferol) or chow deficient in vitamin D. Mice on the latter diet developed marked vitamin D-deficiency within 6 weeks, as indicated by serum 25-hydroxyvitamin D3 levels of less than 20 nmol/l (normal: > 100 nmol/l) (Fig. 6A). Similar to calcium deficiency, these mice developed secondary hyperparathyroidism and accelerated bone resorption as indicated by increased serum bone resorption and formation markers (Fig. 6B and C). Importantly, vitamin D deficiency significantly stimulated skeletal tumour growth in a number of different cancer models, including human breast (MDA-MB-231 cells [60], MCF-7 cells [160] and prostate cancer (PC3 cells) (Fig. 7) [24,161].

In the same experiments, we inhibited bone resorption by administration of OPG to provide further evidence that accelerated bone resorption was indeed responsible for the enhanced tumour growth. Mice on OPG maintained normal calcium levels at the expense of further increases in PTH levels. However, tumour growth was significantly reduced or even abolished by OPG treatment independent of whether the animals were fed a normal diet, or a chow deficient in either calcium or vitamin D [24,60,158–161]. In contrast, when breast or prostate cancer cells were implanted in the subcutaneous soft tissues away from bone, tumours grew similarly in all test groups (controls and calcium or vitamin D deficient mice), indicating that the effects of vitamin D or calcium deficiency on skeletal tumour growth are not systemic but related to changes in the bone microenvironment [24,60,158–161]. Taken together, it
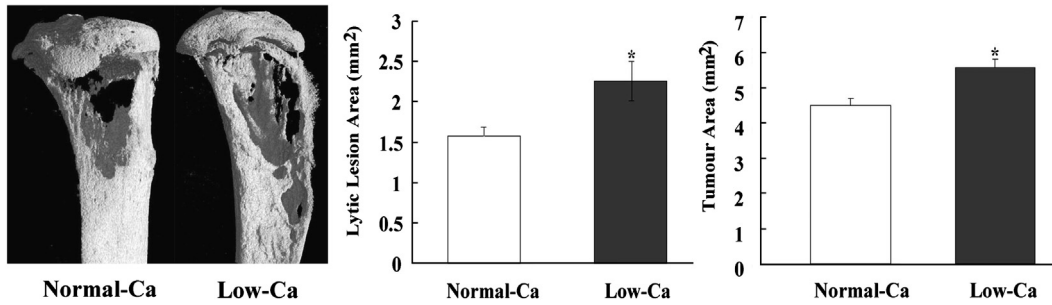
Fig. 5. Low dietary calcium promotes breast cancer growth in bone. Mice fed a low calcium diet and injected intratibially with breast cancer MDA-MB-231 cells develop larger lytic lesions (left and centre) and larger tumours (right) compared to mice on a normal diet. *$p < 0.01$.
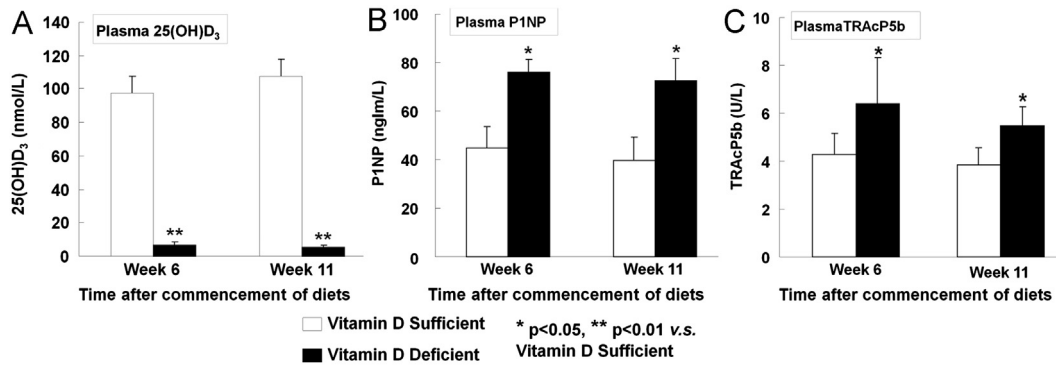From Ref. [158] with permission from the Publisher.

Fig. 6. Biochemical assessment of mice receiving vitamin D deficient or vitamin D sufficient diets. (A) Plasma 25(OH)D levels are profoundly reduced at 6 and 11 weeks. (B and C) Plasma levels of PINP and TRAcP5b were significantly increased in vitamin D deficient mice at week 6. At week 11, plasma PINP levels were still significantly higher in vitamin D deficient compared to vitamin D sufficient mice. There was no difference between TRAcP5b levels.
Data are shown as mean $\pm$ SD for group sizes of $n = 9$.
*, $P < 0.05$, **, $P < 0.01$, compared to vitamin D sufficient mice.
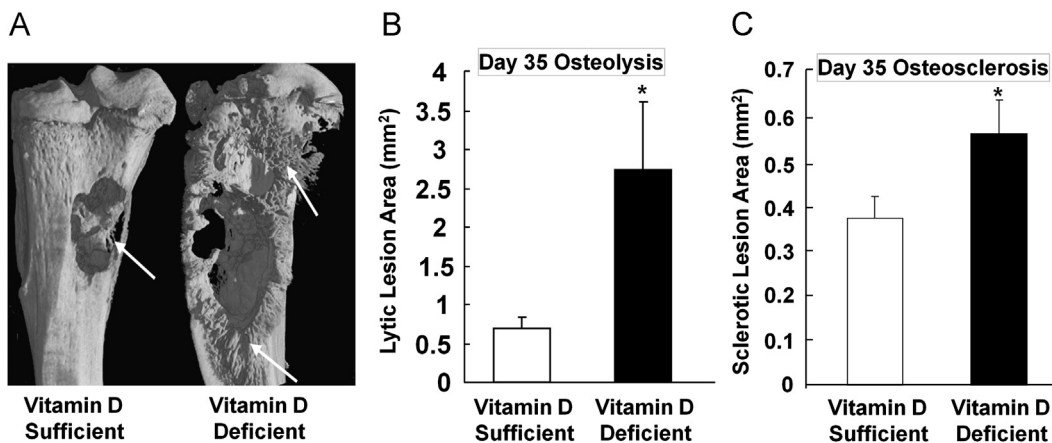From Ref. [161] with permission from the Publisher.

Fig. 7. Radiographic assessment of osteolytic and osteosclerotic lesions in vitamin D sufficient and vitamin D deficient mice. Vitamin D deficient mice had developed significantly larger osteolytic (B) and osteosclerotic lesions (C) than vitamin D sufficient mice (arrows indicate sclerotic lesions, (A)), when implanted with prostate cancer PC-3 cell into tibiae of mice.
Data are shown as mean $\pm$ SE for group sizes of $n = 9$.
*, $P < 0.05$, compared to vitamin D sufficient mice.
From Ref. [161] with permission from the Publisher.

seems clear that accelerated bone turnover, and particularly increased bone resorption are the dominant factors in promoting breast and prostate cancer growth in bone. These results are consistent with the concept that the enhancement of a "vicious cycle" by increased bone resorption supports tumour growth in bone [7,10,12], which further lays a solid ground for anti-resorptive treatment targeting the bone microenvironment for patients who have bone metastasis.

These studies directly link vitamin D deficiency to enhanced tumour expansion in bone metastatic growth, providing support for a causal association between low vitamin D status and enhanced breast and prostate cancer progression as observed in clinical observational studies. These results also provide a clinical and therapeutic rational for maintaining vitamin D sufficiency, or correcting vitamin D deficiency in patients with breast or prostate cancer-induced bone metastases.

5. The role of other bone marrow cells in metastatic cancer growth

Other cell types also that take part in the regulation of the bone microenvironment, including myeloid and immune cells (T cells), platelets, bone marrow endothelial and haematopoietic cells, as well as bone marrow-derived mesenchymal stem cells [3,162]. Some of these cells are likely to participate in the creation of the pre-metastatic niche [3,162].

Haematopoietic cells (other than osteoclasts) have the ability to potentially affect bone metabolism, and in particular bone resorption. For example, T cells produce osteoclast-activating factors such as RANKL, tumour necrosis factor (TNF) and TGF-β [91,163,164], It is via this link that these cells may influence cancer growth in bone. Furthermore, tumour cells are able to activate platelets to release lysophosphatidic acid (LPA), which in turn promotes breast cancer growth and skeletal metastasis in mice via production of IL-6 and IL-8, again potentially augmenting the vicious cycle [67].

In addition, myeloid-derived suppressor cells (MDSCs), platelets, bone marrow endothelial and haematopoietic cells as well as bone marrow-derived mesenchymal stem cells may all be involved in tumour neovascularisation [162]. These cells may interact with other bone cells at various levels and participate in the process of bone metastasis [3,162]. While most of these events affect tumour growth by changing the bone microenvironment, some may have additional effects on cancer cell metastasis by co-operating with osteoblasts, osteocytes and osteoclasts in creating what is known as the pre-metastatic niche.

5.1. The pre-metastatic niche

The vicious cycle, with its associated changes in the bone microenvironment, has been extremely useful in elucidating some of the mechanisms that support established cancer metastases in bone [7,8]. However, being mono-directional and depending on three different cell types to become, and remain activated, the model is less suitable to explain tumour growth at early stages of the metastatic process. It is conceivable that such growth kinetics warrant additional pathways in the form of amplifying elements, which would initiate, sustain and accelerate cell growth and expansion within the bone environment. Different modes of action to achieve amplification could involve direct communication between tumour cells and other bone cells including osteoclasts, osteoblasts and other bone marrow cells, via the various signalling pathways.

Recent advances in preclinical melanoma and lung cancer studies have demonstrated that the bone microenvironment may act as a pre-metastatic niche, through which the primary tumour is able to prime distant organs to become receptive to metastasising tumour cells early during tumourigenesis. For example, vascular endothelial growth factor receptor 1 (VEGFR1)-positive bone marrow-derived haematopoietic progenitor cells are able to travel to the sites of future metastasis before tumour cell arrival to facilitate tumour cell metastasis by increasing production of fibronectin or inflammatory chemoattractants in tumour target sites [165–168]. Bone is rich in the chemokine SDF1 and its neutralisation has been reported to reduce prostate cancer metastasis to bone [169].

In the context of the bone microenvironment, the pre-metastatic niche may function via endocrine-like actions. Perhaps similar to the so-called priming in organs such as lung, primary tumours may be able to set up a pre-conditioning microenvironment through the production of circulating factors, which signal to various cells in the bone microenvironment. In this way the primary tumour could make the bone microenvironment conducive to tumour localization and colonization. There are a few

tumour derived factors such as PTHrP [71,170], heparanase [171] and osteopontin [172,173] which have been reported to increase bone resorption and promote tumour formation. Intriguingly, matrix metalloproteinase (MMP) production from osteoclasts can also support prostate cancer skeletal metastasis [174]. However, the concept of the pre-metastatic niche itself as a potential therapeutic target requires further investigation but is provoking interest in the field.

Furthermore, the possibility of direct crosstalk between tumour cells and osteoblasts has not been well explored. Direct interactions, between these cells could short-cut the vicious cycle and also provide mechanisms relevant to initiation and/or amplification of cancer growth in bone.

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