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Review

Skeletal metastasis: treatments, mouse models, and the Wnt signaling

Kenneth C. Valkenburg[1], Matthew R. Steensma[1, 2], Bart O. Williams[1] and Zhendong Zhong[1]

Abstract

Skeletal metastases result in significant morbidity and mortality. This is particularly true of cancers with a strong predilection for the bone, such as breast, prostate, and lung cancers. There is currently no reliable cure for skeletal metastasis, and palliative therapy options are limited. The Wnt signaling pathway has been found to play an integral role in the process of skeletal metastasis and may be an important clinical target. Several experimental models of skeletal metastasis have been used to find new biomarkers and test new treatments. In this review, we discuss pathologic process of bone metastasis, the roles of the Wnt signaling, and the available experimental models and treatments.

Key words Cancer, skeletal metastasis, therapy, Wnt signaling, mouse models

Skeletal Metastasis

Five-year survival rates for most patients with localized cancers have risen consistently over the past few decades, and cancer metastasis patients are also surviving longer, partially due to advanced treatments, such as treatments with the targeted agent trastuzumab in breast cancer[1]. However, once cancer spreads to the bone, it typically cannot be cured, accounting for significant morbidity and mortality. Skeletal metastasis is common in several of the most prevalent cancers, including prostate, breast, and lung cancers. In fact, 80% of all skeletal metastatic lesions come from one of these primary sites[2]. These three types of cancer account for approximately 43% of all cancer cases in the United States, and they cause approximately 40% of all cancer-related deaths[1]. Recent data support a high rate of hospitalization for patients afflicted by metastatic bone disease, confirming that skeletal-related events also lead to increased use of health care resource[3].

In a healthy person, bone turnover is precisely regulated and occurs on a constant basis. Osteoblasts, which originate from mesenchymal stem cells and eventually differentiate into osteocytes, play a role in bone formation. Osteoblasts and osteocytes also secrete factors that stimulate osteoclastogenesis from hematopoietic precursors; one such factor is receptor activator of nuclear factor kappa B ligand (RANKL) which binds to its receptor RANK, the master osteoclastogenic cytokine[4]. Activated osteoclasts resorb the bones. Osteoprotegerin (OPG) regulates this process and is secreted by osteoblasts and osteocytes. OPG acts as a decoy receptor for RANKL, thereby acting as a competitive inhibitor. In skeletal metastasis, tumor cells disrupt the delicate homeostasis of bone formation and resorption through the secretion of catabolic and/or anabolic factors that uncouple these functions.

The complex process of metastasis consists of several steps[5]. Primary tumor cells become pre-metastatic when they exhibit unlimited proliferation, evasion of apoptosis, and have access to a new supply of blood vessels (angiogenesis)[6]. These cells also display physical changes, such as dysplasia and karyomegaly. Tumor cells must then acquire a metastatic phenotype, generally through mutation or alternative activation of proinvasive molecular pathways[7]. This process is usually referred to as an epithelial-to-mesenchymal transition (EMT): epithelial-like cells, which are well-organized, fully differentiated, and immobile develop into mesenchymal-like cells, which do not organize, are undifferentiated, and are mobile. Tumor cells then intravasate into surrounding blood vessels. Once in the bloodstream, tumor cells that survive anchorage independence and immune surveillance reach new organs, where they extravasate from blood vessels and initiate a metastatic lesion. Angiogenesis is important for the growth and maintenance of the tumor

Authors' Affiliations: [1]Center for Skeletal Disease Research, Van Andel Research Institute, 333 Bostwick Ave. NE, Grand Rapids, MI 49503, USA; [2]Orthopedic Oncology, Spectrum Health, 145 Michigan St. NE, Grand Rapids, MI 49503, USA.

Corresponding Authors:

Bart O. Williams, Center for Skeletal Disease Research, Van Andel Research Institute, 333 Bostwick Ave. NE, Grand Rapids, MI 49503, USA. Email: bart.williams@vai.org.

Zhendong Zhong, Van Andel Research Institute, Grand Rapids, MI 49503, USA. Email: alex.zhong@vai.org or zazhong@ucdavis.edu.

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in a new microenvironment. Depending on the type of primary tumor, metastatic lesions often occur in specific organs; bone metastasis occurs most commonly from breast, prostate, and lung cancers.

Metastasis to the bones involves tumor cells (the "seed") invading the bone cavity (the "soil"[8]) and causing a so-called vicious cycle[9]. Upon invasion into the bone, tumor cells secrete factors that affect both osteoblasts and osteoclasts, disrupting normal homeostasis (Figure 1). In many cases of skeletal metastasis, particularly from breast and lung cancers, the lesions are prone to be osteolytic, resorbing more bone tissue than that is being formed. Breast cancer skeletal metastatic cells have up-regulated expression of matrix metallopeptidase-1 (MMP-1), interleukin-11 (IL-11), and C-X-C chemokine receptor 4 (CXCR4), which favor osteoclastogenesis and increase bone resorption[10]. This results in lower bone mass, pain, increased risk of fracture, and other symptoms. In other cases, particularly in most prostate cancer metastases, lesions are prone to be osteoblastic, forming more bone tissue than that is being resorbed. Such lesions—also known as osteosclerotic or osteoblastic lesions—contain poorly organized collagen fibers that have a woven appearance and weak structure, which also results in pain and increased fracture risk[11].

Prostate cancer cells generally up-regulate the Wnt/β-catenin pathway, bone morphogenetic proteins (BMPs), platelet-derived growth factor (PDGF), and endothelin-1 (ET-1), which results in a more pro-osteoblastic phenotype[7]. It is becoming more widely recognized that most cases of skeletal metastasis have both osteolytic and osteoblastic components and that osteolysis is likely required for advancement of osteoblastic disease[9,11]. The bone resorption marker N-telopeptide type I collagen is increased in all skeletal metastases, regardless of whether they appear to be osteoblastic or osteolytic, indicating that osteoclastogenesis and bone resorption occur in all skeletal metastasis[12]. Much more work remains to be done to elucidate the complexity of skeletal metastasis and to identify molecular targets that can retain normal bone homeostasis, while simultaneously killing the invading tumor cells.

The affinity that certain cancers have for the bone is still an area under considerable investigation. Studies

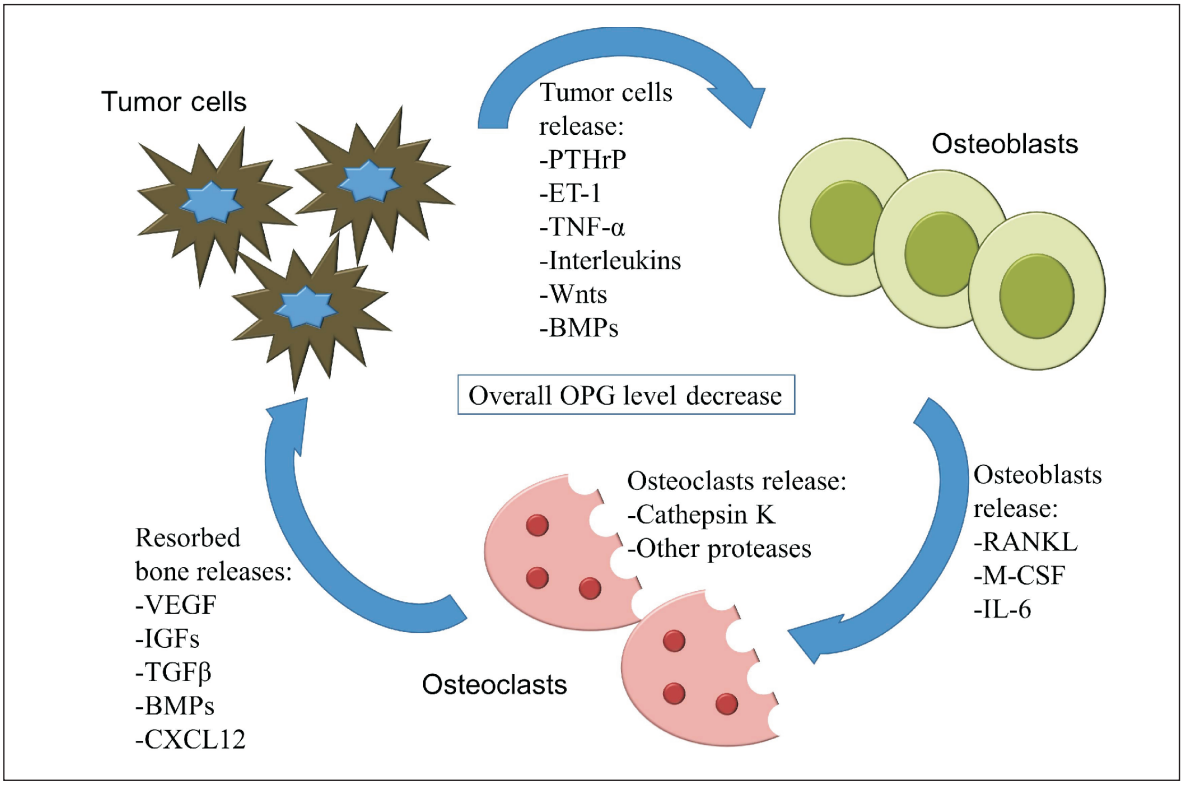

Figure 1. **The "vicious cycle" takes place within the tumor-bone microenvironment.** Tumor cells secrete factors that stimulate osteoblast activation and bone formation. Osteoblasts release RANK ligand and other factors that stimulate osteoclast activation and resorption of the bones. This allows for the release of growth factors that stimulate tumor growth and maintenance. These factors and their concentrations can change throughout the process of metastasis and can differ from cancer to cancer, which results in a variable balance of bone formation and resorption. PTHrP, parathyroid hormone-related protein; ET-1, endothelin-1; TNF-α, tumor necrosis factor alpha; BMP, bone morphogenetic protein; RANKL, receptor activator of nuclear kappa-B ligand; M-CSF, macrophage colony-stimulating factor; IL-6, interleukin-6; VEGF, vascular endothelial growth factor; IGF, insulin-like growth factor; TGFβ, transforming growth factor beta; CXCL12, chemokine C-X-C motif ligand 12; OPG, osteoprotegerin.

suggest that tumor cells have up-regulated expression of cell surface proteins such as CXCR4 or certain integrins, and therefore have a higher preference to home to the bone[13,14]. Cell adhesion molecules regulate circulating tumor cell interactions with endothelial cells in the blood vessels of specific organs[15]. Resorbed bone also releases a wealth of growth factors that induce circulating tumor cell homing, tumor growth, angiogenesis, and maintenance. Additionally, endocrine factors such as heparanase, osteopontin, and matrix metalloproteases are produced by primary tumors and go into circulation before actual metastasis occurs, setting up a "pre-metastatic niche"[16-19]. Alternatively, tumor cells may use strategies similar to those of hematopoietic stem cells to home to the bone; recent studies have shown that prostate tumor cells compete for the niche of such stem cells in the bones[20].

In prostate cancer cases, most men present with non-metastatic disease; only about 3% of men are initially diagnosed with metastatic disease, but that number jumps to 12% upon further follow-up[21]. However, skeletal metastases are found in approximately 90% of prostate cancer patients who succumbed to their disease[2,22]. In breast cancer, skeletal metastases are similarly found in a small proportion of initially diagnosed patients, but approximately 43% developed metastasis upon follow-up[23]. Upon autopsy, skeletal metastases were found in 71% of breast cancer patients and 41% of lung cancer patients[24,25]. Five-year survival rates decrease dramatically in patients with metastatic disease when compared with patients with local disease (Table 1). In prostate and breast cancers, skeletal metastasis is so prevalent that it is probable that the majority of the tumor burden of patients who succumb to the disease will reside in the bone[9]. Skeletal-related events are defined as pathologic fractures, spinal cord compression, or bone pain requiring palliative radiotherapy and orthopedic surgery[23]. Pain is especially common in the load-bearing bones such as the femoral neck, pelvis, and vertebrae. Approximately 20% of prostate cancer patients with bone metastasis are treated for pathologic fractures[11]. Skeletal metastasis can cause compression syndromes of nerve roots and the spinal cord, inducing systemic and head pain. Osteolytic bone lesions induce hypercalcemia by overwhelming the calcium homeostatic mechanisms in the body; thus, extracellular fluid calcium is filtered out and excreted by the kidneys[9]. Phosphate and other metabolic imbalances can occur[9]. All these symptoms require treatment and palliative care, adding to the burden of the patient and increasing the cost of health care dramatically.

Current and Potential Therapies for Skeletal Metastasis

Several treatment options exist for patients afflicted with skeletal metastasis. Surgical treatment with curative intent is recommended for certain oligometastatic presentations of skeletal metastasis, but few actual cures are achieved. Traditional treatment methods include chemotherapy, anti-resorptive medications, radiotherapy, and surgical stabilization. Other palliative options include narcotic medications and activity restriction.

Traditional chemotherapy, such as docetaxel, is less effective against bone metastases. A relatively new strategy for treating bone metastasis is targeting the bone microenvironment itself. Table 2 contains an extensive list of potential and currently available treatments for skeletal metastasis. Many of these treatments can target both the bone microenvironment and tumor cells. The most commonly applied strategies for treating bone metastasis are surgery, radiotherapy, hormone ablation, anti-resorptive strategies, and targeted therapies.

Surgery

In many cases of bone metastasis, surgery is used to treat impending or displaced pathologic fractures. Pathologic fractures cause significant morbidity and often interrupt ongoing treatment efforts. To this end, the primary focus of the orthopedic surgeons performing these surgeries is to stabilize the bones in the extremities, such as the pelvis, spine, or femur. Stabilization is accomplished with prostheses, arthroplasty, plates, or intramedullary fixation devices[26]. Surgery is effective in improving pain control and mobility, and facilitates subsequent efforts to employ radiation and chemotherapy[27]. The survival rate of patients with skeletal metastasis treated with surgery

Table 1. Five-year survival rates by stage at diagnosis, 1999–2006

Cancer type	Local disease[a]	Metastatic disease[b]	Typical sites of metastasis
Prostate cancer	100%	30%	Bone
Breast cancer	98%	23%	Bone, lungs, liver, brain
Lung cancer	53%	4%	Bone, brain, liver, adrenal gland

[a]Local disease refers to invasive malignant cancer that is entirely confined to the primary organ.
[b]Metastatic disease refers to a malignant cancer that has spread to any part of the body outside of the primary tumor.
Statistics were taken from the American Cancer Society[1].

Table 2. Current and potential targeted therapies for skeletal metastasis

Mechanism of action	Example therapeutic(s)	Stage of clinical availability	Reference(s)
Osteoclast apoptosis	Bisphosphonates	Available	[55]
RANKL inhibition	Denosumab	Available	[63]
Microtubule inhibition	Docetaxel	Available	[177]
	Cabazitaxel	Available	[178]
EGFR/ERRB2 inhibitor	Lapatinib	Available	[179]
Cancer immunotherapy	Sipuleucel-T / ipilimumab	Available	[180]
DNA synthesis inhibition	Gemcitabine / satraplatin / pemetrexed disodium	Available	[181–183]
VEGF inhibition	Bevacizumab / sorafenib / sunitinib	Available	[184–186]
CYP17 inhibition	Abiraterone acetate	Available	[44]
Competitive binding to RANK	Recombinant osteoprotegrin	Available	[187,188]
Kinase inhibition	Crizontinib	Clinical trials	[189]
MET/VEGFR2 inhibition	Cabazantinib	Clinical trials	[95–97]
Src inhibition	Dasatinib / saracatinib / bosutinib	Clinical trials	[99]
Vitamin D analogues	Calcitriol / EB1089	Clinical trials	[190,191]
Proteasome inhibition	Bortezomib	Clinical trials	[192,193]
Radiopharmaceuticals	Radium-223 / strontium-89	Clinical trials	[194]
Endothelin A inhibition	ZD4054 (Zibotentan)	Clinical trials	[195]
DKK-1 inhibition	DKK-1 antibodies	Clinical trials	[158]
PARP inhibition	Veliparib	Discovery	[196]
Integrin inhibition	Cilengitide	Discovery	[197]
PTHrP inhibition	PTHrP antibodies	Discovery	[81,82]
Cathepsin K inhibition	Balicatib / odanacatib	Discovery	[72–74]
Cathepsin B inhibition	CA-074	Discovery	[75]
TGFβ inhibition	LY2109761 / Ad.sTβRFc / Ki26894	Discovery	[90,91,93]
mTOR inhibition	Rapamycin	Discovery	[198,199]
MMP inhibition	Batimastat	Discovery	[200,201]
Organic compounds	Plumbagin	Discovery	[202]
IL-6 inhibition	Tocilizumab / elsilimobab	Discovery	[203,204]
Activin-A inhibition	ActRIIA.muFc	Discovery	[205]
Platelet inhibition	XV454	Discovery	[206,207]
Wnt inhibition	Lrp6 antibodies / IWP-2 / C59	Discovery	[154,208–210]

RANKL, receptor activator of nuclear factor kappa B ligand; EGFR/ERRB2, epidermal growth factor receptor/estrogen related receptor beta; VEGF, vascular endothelial growth factor; CYP17, cytochrome P450 17α; RANK, receptor activator of nuclear factor; VEGFR, VEGF receptor; DKK, dickkopf; PARP, poly (ADP-ribose) polymerase; PHTrP, parathyroid hormone-related protein; TGFβ, transforming growth factor beta; mTOR, mammalian target of rapamycin; MMP, matrix metallopeptidase; IL-6, interleukin-6.

was approximately 40% after 1 year, 30% after 2 years, and 20% after 3 years[28]. In most cases of skeletal metastases arising from breast cancer, prostate cancer, lung cancer, or melanoma, surgery is not performed with the intent to cure, but an indirect survival benefit may be derived from enhanced function and performance status[29,30]. Surgery has also been reported to prolong survival for patients with oligometastatic presentation of renal cell carcinoma, thyroid carcinoma, and myeloma[31,32].

Radiotherapy

Radiotherapy is an effective treatment for skeletal metastasis in terms of preventing impending fractures, controlling pain, and restoring function[33,34]. In rare cases, radiotherapy has been shown to have a curative effect on bone lesions. Radiation is often used as a stand-alone treatment for radiosensitive cancer types, and as an adjuvant to surgical stabilization of pathologic fractures[35]. External beam radiation therapy has been the mainstay in past years of radiotherapy, meaning that tumor cells and normal cells receive the same amount of radiation in the region being treated. Thus, effort has been placed into more efficiently targeting tumor cells with radiation. In particular, one study showed that image-guided high-dose radiation was an effective treatment, in the absence of surgery, and that patients were typically cancer-free 3 years after treatment[36]. In addition, spine stereotactic body radiotherapy allows for delivery of a

high dose to metastatic regions without inducing severe adverse effects on adjacent spinal cord[37]. A promising new treatment is alpha-pharmaceuticals, radionuclides that emit alpha particles, which deliver a more intense radiation than beta-emitting radiopharmaceuticals, in a targeted fashion[38]. Radium-223 is the first alpha-emitting radiopharmaceutical to reach clinical trials and has been shown in phases I and II trials to improve survival in patients with skeletal metastases from prostate cancer[38].

Hormone ablation

Hormone ablation is an efficient strategy for shrinking breast and prostate cancers; both cancers depend on hormones for tumor growth and maintenance. For breast cancer, tamoxifen (Nolvadex), which blocks estrogen binding to the estrogen receptor[39], is the standard of care following chemotherapy. Alternatively, aromatase inhibitors (arimidex, aromasin, and femara are currently approved) inhibit estrogen synthesis, thereby decreasing the concentration of estrogen in the body and inhibiting the primary tumor's ability to grow and metastasize. Aromatase inhibitors are recommended for breast cancer treatment either together with tamoxifen or as an extended therapy after tamoxifen[39]. These estrogen-inhibiting strategies have increased the life span of breast cancer patients and have reduced recurrence[39]. No difference in survival has been reported between tamoxifen and aromatase inhibitor treatment[39].

In prostate cancer, lutenizing hormone-releasing hormone (LHRH) agonists inhibit testosterone production, which has been shown to shrink prostate cancer and increase life span[40]. However, most patients become castration-resistant, and many of these patients have increased skeletal-related events[41]. Deferred androgen deprivation therapy may be ideal; the time at which to initiate androgen deprivation therapy is still controversial[42].

One promising drug approved by the Food and Drug Ad-ministration (FDA) in 2011 for treatment of metastatic castration-resistant prostate cancer is abiraterone (Zytiga). Abiraterone inhibits 17 α-hydroxylase/C17,20 lyase (CYP17A1), which is re-sponsible for the conversion of progesterone and pregnenolone to testosterone precursors. This inhibition of alternate sources of testosterone results in an overall decrease in the amount of circulating testosterone. In the first clinical trial in Britain in 2004, abiraterone induced sustained suppression of testosterone levels[43]. A report in 2011 showed that abiraterone increased overall survival by approximately 4 months in men with metastatic castration-resistant prostate cancer who had previously undergone chemotherapy[44]. A later study showed that it reduced hormone levels not only in the tumor but also in metastatic bone lesions[45].

Although hormone ablation can benefit patients by shrinking their primary tumors, this treatment has been suggested to increase the frequency of skeletal-related events and may in fact promote osteoclastogenesis, thereby "putting fuel on the fire" and increasing bone turnover[46]. Estrogen can inhibit osteoclastogenesis, which may explain why postmenopausal women more commonly suffer from osteoporosis[47]. Also, both estrogen and androgen ablation therapies decrease overall bone mass density, leading to increased risk of fracture independent of metastasis[48-50]. However, studies on the selective estrogen response modulators raloxifene and tamoxifen have shown that there is no increased risk of invasive breast cancer, and in fact the risk is decreased in women with postmenopausal osteoporosis[51,52]. Combined, these data suggest that while hormonal ablation is an important and effective treatment for primary breast and prostate cancers, it may be risky in more aggressive cancers. This topic needs further investigation and clarification.

Anti-resorptive strategies

The theory behind anti-resorptive strategies is that by halting osteoclast-mediated bone resorption, bone turnover is halted, stopping osteolysis and decreasing the size of skeletal metastases. Bisphosphonates are widely used to treat skeletal metastasis and other diseases associated with bone loss, such as osteoporosis and Paget's disease. Bisphosphonates can inhibit bone turnover by causing osteoclast apoptosis in two ways: by acting through toxic metabolites, preventing isoprenylation of small GTP-binding proteins[53]; or by inhibiting farnesyl pyrophosphate synthase, which is essential for stability of the osteoclast cytoskeleton[54,55]. Additionally, bisphosphonates can directly induce cancer cell apoptosis and inhibit pro-osteoclastic gene expression[56]. The most serious adverse effects of bisphosphonates are renal failure and anaphylaxis[57]; two other rare adverse effects are osteonecrosis of the jaw and atypical femur fractures[58]. Table 3 contains a list of available bisphosphonates.

Zoledronic acid (ZA; also known as zolendronate or Zometa) is the most commonly used and most potent bisphosphonate on the market[59]. It can be used at a lower dose (4 mg) than pamidronate (90 mg), another approved bisphosphonate[57]. Treatment with bisphosphonates decreased the number of

Table 3. Currently available bisphosphonates

Bisphosphonate	Commercial name	Dosage route	Reference
Zoledronic acid	Zometa/ Reclast	Intravenous	[57]
Pamidronate disodium	Aredia	Intravenous	[211]
Alendronate sodium	Fosamax	Oral	[212]
Etidronate disodium	Didronel	Oral	[213]
Ibandronate sodium	Boniva	Oral	[214]
Risedronate sodium	Actonel	Oral	[215]
Tiludronate disodium	Skelid	Oral	[216]

skeletal-related events and lengthened the time-to-first-skeletal-related event in breast and prostate cancers, as well as in multiple myeloma[57]. Despite their wide use, bisphosphonates have no overall survival or disease-free survival advantage and do not fully inhibit skeletal metastasis or its morbidity. The lack of survival advantage could be because metastatic tumors have already caused debilitating damage in the bone before bisphosphonates are even used for treatment. ZA is now undergoing clinical trials with various other adjuvant therapies[11]. Another interesting use of bisphosphonates was highlighted in a recent study that used alendronate, conjugated to a peptidomimetic ligand against activated integrin α4β1, to serve as a bone-seeking agent to direct the therapeutic compound to the bone[60].

Targeted anti-RANKL therapy is evolving as an anti-resorptive strategy. Denosumab (also known as Xgeva) is a monoclonal antibody that competitively binds RANKL, resulting in potent inhibition of bone resorption[61]. Although mechanistically similar to bisphosphonates, denosumab may be more potent in preventing skeletal-related events in the setting of skeletal metastasis[62]. Denosumab has been shown to decrease the rate of skeletal-related events from 18% (ZA) to 7%[63]. Treatment with denosumab decreased the risk of renal toxicity as compared with bisphosphonates. More data are needed to determine whether anti-RANKL therapy is more efficacious than other anti-resorptive strategies in skeletal metastasis and to determine its relative cost-effectiveness[64]. Denosumab is in clinical trials to determine its effectiveness in combination with other chemotherapies. However, long-term adverse effects could be important; recent studies have shown that patients treated with bisphosphonates for more than 5 years had a higher risk of suffering atypical femur fractures, possibly caused by suppressed, bone turnover-related microdamage[65]. While denosumab has been approved by the FDA for use in non-metastatic prostate cancer patients to reduce the risk of fracture, it was recently rejected for treatment of bone metastasis in prostate cancer patients because it did not significantly increase overall survival[66].

Other RANKL inhibitors, such as RANK-Fc and OPG-Fc, have been developed for treatment of skeletal metastasis. Several animal models that mimic advanced prostate cancer, breast cancer, or non–small cell lung cancer, representing both osteolytic and osteoblastic skeletal lesions, were used to determine whether these RANKL inhibitors were practical and effective in inhibiting bone metastases[67-69]. In a murine model of human A431 epidermoid carcinoma bone metastasis, OPG-Fc inhibited RANKL, suppressed tumor-induced osteolysis and ultimately inhibited tumor cell growth in the bone, but there was no effect on the subcutaneous growth of the tumor[70]. The bone metastasis suppression effect of these RANKL inhibitors seems to be more prominent if combined with therapeutics that also target the tumor itself[71].

Although other drugs against bone metastasis have used the same strategy of inhibiting osteoclasts or bone turnover, none have been as successful in clinical trials as bisphosphonates and denosumab. Cathepsin K is a protease involved in bone resorption, and pre-clinical studies inhibiting cathepsin K have shown promise in ablating skeletal metastasis[72]. Balicatib inhibits cathepsin K and showed potential to inhibit osteolysis and regain bone mass, but it was withdrawn from clinical trials due to safety issues[73]; Odanacatib, another cathepsin K inhibitor, was also withdrawn from clinical trials for breast cancer bone metastasis for safety concerns[11,74]. Most recently, intraperitoneal injections of the cathepsin B inhibitor CA-074 into mice in the 4T1.2 model (a breast cancer model that exhibits spontaneous bone metastases) reduced bone metastases significantly ($P < 0.05$), indicating an important role for cathepsin B in late-stage skeletal metastasis[75].

Targeted therapies

A promising strategy for treating cancer as a whole, as well as for treating skeletal metastasis, is to focus on specifically up-regulated oncogenic molecular pathways. The principle of this strategy is to target and inhibit pro-metastatic genes to stop or reduce the ability

of cancer cells to metastasize. In this section, we will highlight some hopeful molecular targets in the skeletal metastasis field.

PTHrP

Parathyroid hormone-related protein (PTHrP) has important roles during the development of several organs and is dys-regulated in several types of cancer. The expression of PTHrP correlates with prostate cancer progression[76], and PTHrP can increase prostate cancer cell migration and invasion[77]. EB1089, a potent agonist of the vitamin D signaling, can reverse these effects by down-regulating PTHrP[77]. Furthermore, PTHrP increases osteoblastic cell survival through the vascular endothelial growth factor receptor 2 (VEGFR2) pathway, again indicating its importance in prostate cancer bone metastases[78]. PTHrP has also been linked to breast and lung cancer skeletal metastases[79-81]. Humanized monoclonal antibodies that block PTHrP binding to its receptor reduce bone metastasis in a breast cancer model[82] and in a lung cancer model[81].

TGFβ

Transforming growth factor beta (TGFβ) is important during development, and its up-regulation has long been known as a crucial factor in promoting cancer metastasis[83,84]. TGFβ is the most abundant cytokine in the bone matrix[85,86]. Its release during bone resorption helps induce bone marrow stromal cells, serving to couple bone resorption and formation during development and bone remodeling[87,88]. Because TGFβ can induce EMT in tumor cells, it likely has a major role in metastasis, and as such represents a major target for treatment[89]. Ki26894, a novel inhibitor of TGFβ, inhibited the ability of breast tumor cells to secrete PTHrP, reduced the frequency of bone metastases, and increased the life span of mice injected with breast cancer cells[90]. When the dominant-negative mutant TβRIIΔcyt, which is unresponsive to TGFβ, was overexpressed in breast cancer cells injected into mice, skeletal metastasis and bone destruction were reduced, and overall survival increased[91]. Interestingly, this phenotype was shown to be due to a decrease in PTHrP expression[91]. The TGFβ signaling is a biomarker of poor prognosis in prostate cancer[92]. The TGFβ receptor type I (TGFβRI) inhibitor LY2109761 not only decreased tumor growth in the bone but increased bone mass[93]. Finally, oncolytic viruses expressing TGFβ receptor type II (TGFβRII) fused to human Fc (Ad.sTβRFc) reduced skeletal metastasis and associated hypercalcemia, indicating a therapeutic potential[94].

MET/VEGF

Cabozantinib (XL 184) has shown promise in clinical trials. It inhibits the activity of several receptor kinases, most potently hepatocyte growth factor receptor (MET) and vascular endothelial growth factor receptor (VEGFR), which are functionally linked[95,96]. Studies have suggested that bone formation and angiogenesis are coupled, indicating that VEGF inhibition would be an important strategy for halting skeletal metastasis[83]. In one clinical trial in which 62 patients underwent cabozantinib treatment, 85% achieved complete or partial resolution of metastatic lesions upon bone scan[97]. Cabozantinib is now in phase III clinical trials for castration-resistant prostate cancer and will be used in at least 12 other clinical trials for other tumor types.

There are other molecular targets that also have promise in this field. Notably, the inhibition of Src kinase and mammalian target of rapamycin (mTOR) has each been shown to have positive effects on reducing bone metastasis[98,99]. These and other targets can be found in recent reviews[41,100-103].

Summary

In each of these pre-clinical models or clinical cases, investigators have reported that inhibition of a specific molecular pathway reduced the frequency of skeletal metastasis, increased patient survival, or both. However, there is still no standard treatment for patients suffering from painful and lethal skeletal metastasis. This suggests that skeletal metastasis is more complex than originally imagined and that we as a research community have not yet identified appropriate targets or combinations of therapies to successfully manage and cure this disease. In the past ten or more years, there have been 8 negative phase III clinical trials for increasing the overall survival of men with skeletal metastatic prostate cancer. This has continued with the failure of denosumab to increase life span. Strategies that target only one cell type or microenvironment, such as the tumor or the bone niche, are not likely to succeed. Accordingly, the need for better experimental models, novel clinical targets, and better use of the knowledge we have is glaringly apparent.

The Wnt Signaling in Skeletal Metastasis

The roles of the Wnt/β-catenin signaling (Figure 2) in tumorigenesis, bone development, and skeletal metastasis have been extensively studied. The Wnt signaling is important in prostate cancer tumorigenesis[104-109] and is positively correlated with the progression of prostate cancer[110-114]. While breast cancer tumorigenesis and poor prognosis have been associated with increased Wnt signaling[115-119], there is less evidence

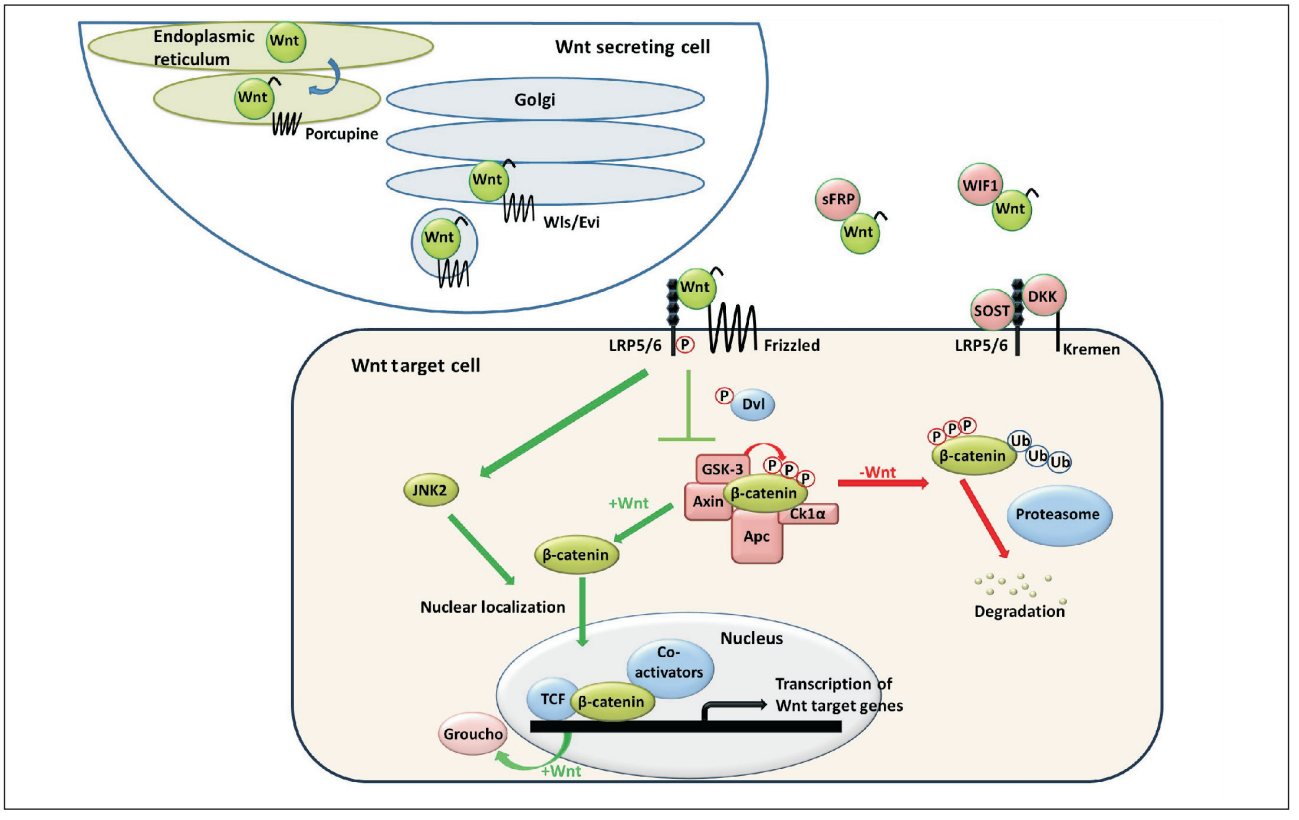

Figure 2. **The Wnt/β-catenin signaling pathway. Wnt is secreted, binds to receptors, and causes β-catenin to accumulate in the cytosol and subsequently migrate to the nucleus to activate transcription of Wnt target genes.** Wls/Evi, Wntless/evenness interrupted; sFRP, secreted frizzled-related protein; WIF1, Wnt inhibitory factor 1; SOST, sclerostin; DKK1, Dickkopf 1; LRP5/6, low-density lipoprotein receptor-related protein 5/6; Dvl, disheveled; GSK3, glycogen synthase kinase 3; Apc, adenomatous polyposis coli; CK1α, casein kinase 1 alpha; JNK2, c-Jun N-terminal kinase 2; TCF, T-cell factor. The authors have published this figure previously in "Wnt/β-catenin signaling in normal and cancer stem cells" [Cancer, 2011,3:2050–2079] and have got the permission to republish this figure.

to suggest that active Wnt signaling induces skeletal metastasis[120]. However, recent studies have shown an association between increased β-catenin–independent Wnt5a expression and brain metastasis[121]. There is also strong evidence that the Wnt signaling is crucial in the development of the breast and prostate glands[122]. Lung tumorigenesis is also associated with up-regulated Wnt signaling[123-126]. Cigarette smoke, which increases the risk for lung cancer substantially, may even activate the Wnt signaling[127].

One of the well-established roles of the Wnt signaling is the activation of the osteoblast lineage from mesenchymal stem cells and the maintenance of bone homeostasis[128]. Based on studies using genetically engineered mice, the downstream effector molecules in the canonical Wnt pathway (such as β-catenin) and the endogenous inhibitors of the Wnt signaling [such as Dickkopf-1 (Dkk1) and sclerostin] play profound regulatory roles by direct action on osteoblasts or indirect action on osteoclasts[129-132]. In addition, Wnt proteins seem to have a role in regeneration during bone healing[133]. These data suggest that the Wnt signaling is crucial for bone development and turnover and, as such, is implicated in the interruption of bone homeostasis during skeletal metastasis and could be an important player in the development and progression of these lesions.

In prostate cancer, the Wnt signaling is involved in osteoblastic bone metastasis. Wnt1 and β-catenin expression levels are low in normal prostate cells but high in prostate cancer cells; high levels are seen in 85% of human skeletal metastases[110]. Two studies in prostate cancer cell lines have shown that inhibition of the Wnt signaling (via overexpression of the endogenous Wnt inhibitory factor WIF1 or knockdown of β-catenin itself) causes a reversal of EMT and the metastatic phenotype[134,135]. Hall et al.[112] showed that knockdown of the Wnt inhibitor Dkk1 in osteolytic PC3 xenografts induced osteoblastic activity. Alternatively, when Dkk1 was overexpressed in osteoblastic C42B4 xenografts, the tumors became highly osteolytic. Taken together, this indicates that induction of the Wnt signaling from prostate cancer tumor cells contributes to osteoblastic

skeletal metastases[112].

Active Wnt signaling induces bone morphogenetic protein activity, and Wnt can induce osteoblastic activity in culture both in BMP-dependent and BMP-independent fashions[136]. Additionally, loss of Dkk1 promotes osteoblastic activity and the expression of BMP4 and BMP6[136]. A study using a canine prostate cancer model showed that Dkk1 overexpression potently inhibited bone growth in a xenograft model, but it also induced primary tumor growth and increased the rate of metastasis[137]. When osteoblast cultures from Lrp5-knockout mice were treated with medium from a prostate tumor cell line capable of inducing osteoblastic metastases *in vivo*, there was no effect, suggesting that at least in this cell line (MDA PCa 2b), prostate cancer–induced osteoblastic activity is mediated by the Wnt receptor Lrp5[138]. These data strongly indicate that the Wnt signaling is active and potentially causative in prostate cancer skeletal metastasis. This idea may contrast with data from genetically engineered mouse models in which activation of the Wnt signaling by knockout of adenomatous polyposis coli (Apc)[107] or direct activation of β-catenin[108] induced carcinoma or prostate intraepithelial neoplasia (PIN), respectively, but did not produce skeletal metastasis. Induction of the Wnt signaling at an early stage may cause non-metastatic prostate cancer, and induction of the Wnt signaling at a late stage may cause osteoblastic metastases.

Breast and lung metastases are often osteolytic, and dys-regulation of the Wnt signaling has a role in bone metastasis in these cancers. Dkk1 expression is up-regulated in osteolytic breast cancer bone metastases, resulting in repression of the Wnt signaling[139,140]. Breast cancer cells treated with Wnt3a-conditioned media caused osteoblastic metastases and RANKL reduction, and this process was blocked by Dkk1-conditioned media from typically osteolytic breast cancer cells[139]. These data indicate that, in contrast to prostate cancer, breast cancer operates via repression of the Wnt signaling rather than induction, thereby producing osteolytic lesions. On the other hand, the Wnt signaling stimulates the motility of breast cancer cells, and the expression of the Wnt inhibitor secreted frizzled-related protein (sFRP1) can inhibit this motility[141]. More work is needed to determine the role of the Wnt signaling in the processes of primary tumorigenesis, EMT, and skeletal metastasis of breast cancer cells.

In lung cancer, the Wnt/TCF4 pathway signature in a cohort of 368 primary tumors was significantly correlated with relapse (P = 0.006)[123]. Additionally, a specific Wnt-responsive gene set was associated with lung cancer metastasis, and expression of a dominant negative version of the Wnt transcription factor TCF4 in a xenograft mouse model resulted in significantly fewer bone metastases[123]. Another group has shown that activation of β-catenin in lung epithelial cells induces EMT; interestingly, this phenotype was reversed after knockdown of Jun N-terminal kinase (JNK1)[142]. Finally, Wnt2 was overexpressed in lung cancer tissue, and when Wnt2 was inhibited with a monoclonal antibody or siRNA, apoptosis occurred in lung cancer cells[143]. These data indicate that aberrant Wnt signaling plays an important role in lung cancer cell survival and skeletal metastasis.

The Wnt signaling is crucial for the proper development of numerous cell and tissue types, as well as for maintenance of adult stem cell populations[144]. Because of this role, the Wnt signaling may contribute to metastasis by causing cells to de-differentiate and become more mesenchymal-like or stem-like[145,146]. The Wnt signaling increases the capacity of mesenchymal stem cells to proliferate and migrate, which indicates that the Wnt signaling is important during tissue regenerative processes, but also suggests that up-regulated Wnt signaling could induce higher migration and invasion of tumor cells having undergone EMT[147]. In addition, the Wnt signaling promotes the self-renewal of prostate stem-like cells and growth of prostaspheres in culture, suggesting that inhibition of the Wnt signaling could have therapeutic value by reducing this stem-like potential[109]. Similar conclusions were drawn from studies of breast and lung cells[120,148,149].

Due to the well-established role of the Wnt signaling in cancer, EMT, and skeletal metastasis, the Wnt pathway represents an excellent target for therapy. The pathway is complex and has many components that may represent therapeutic targets[150]. There are also many mechanisms by which the pathway can be inhibited[122]. A new way of inhibiting the Wnt signaling is through porcupine inhibitors[151]. Porcupine is a membrane-bound O-acetyltransferase (MBOAT) that is required for Wnt ligand palmitoylation and secretion[152]. It is unclear whether porcupine inhibitors will have a substantial effect in cancer, but there are already pre-clinical trials to test that[153]. Several excellent reviews provide insight into the many facets of drugging the Wnt pathway in cancer[154-156].

Because the Wnt signaling can promote both tumorigenesis and osteoblast-mediated bone formation, it is unclear how the Wnt pathway should be modulated as a treatment in skeletal metastasis. Some studies show that promotion of the Wnt signaling via Dkk1 inhibition increased bone formation in osteolytic lesions and decreased osteolysis-related pain and risk of fracture[139,157,158]. Others have shown that inhibition of Dkk1 results in loss of control of the Wnt signaling and promotes proliferation and metastasis of cancer cells[137,159]. The level of the Wnt signaling taking place in the microenvironment of both the primary tumor and skeletal metastasis may be unique for each individual tumor. Therefore, the most likely use of Wnt inhibition will be against tumors in which the Wnt signaling is highly up-regulated, but all indications suggest that the strength and specificity of Wnt inhibition must be strictly regulated

to be of utmost benefit to the patient.

Mouse Models of Skeletal Metastasis

Mouse models have provided many insights into human disease. The ideal mouse model for skeletal metastasis would reproduce all the stages of tumorigenesis and metastasis, including genetic changes, metastatic phenotype of cancer cells, and modifications to the bone microenvironment. Obviously, this is a difficult goal to achieve. Genetically engineered mouse models are generally considered to be an accurate way to mimic human cancer, but despite numerous attempts, there is currently no genetically engineered mouse model that accurately and consistently recapitulates the skeletal metastasis process[146,160].

Consequently, the present paradigm for modeling skeletal metastasis is the xenograft mouse model, in which cells from human cancer cell lines or primary tumors are injected into immune-deficient mice. These cells can form primary tumors that metastasize to the bone and other organs. Several immune-deficient mice are available for such models, including athymic nude, severe combined immune deficient (SCID), non-obese diabetic (NOD-SCID), and NOD-SCID-ILR2Gamma–deficient (NSG) mice[146]. Mouse models of skeletal metastasis can be categorized based on the injection site: orthotopic, intraosseous, intracardiac, intravenous, or subcutaneous (Figure 3). The goal of these models is not only to discover the physiological roles of molecular pathways and of the unique interactions between tumor and the bone but also to determine the effects of potential and novel therapeutics.

Orthotopic injections

Orthotopic prostate injections are commonly done into the ventral or dorsal lobes of the murine prostate, as they are the most readily accessible and most anatomically similar to the human prostate[161]. Mammary orthotopic injections are generally done into the 4th mammary fat pad of a 4- to 5-week-old mouse, which has been surgically cleared of mammary epithelium tissue, leaving behind the fat[160]. Lung orthotopic injections are through the chest and directly into the lung[162,163].

Because skeletal metastases are relatively rare in orthotopic models, such studies are generally centered on the development and growth of primary tumors. This model can be used to study the effect of novel drugs on tumor volume or on the ability of the tumor to invade surrounding stroma or to metastasize to lymph nodes. If skeletal metastasis can be induced using this type

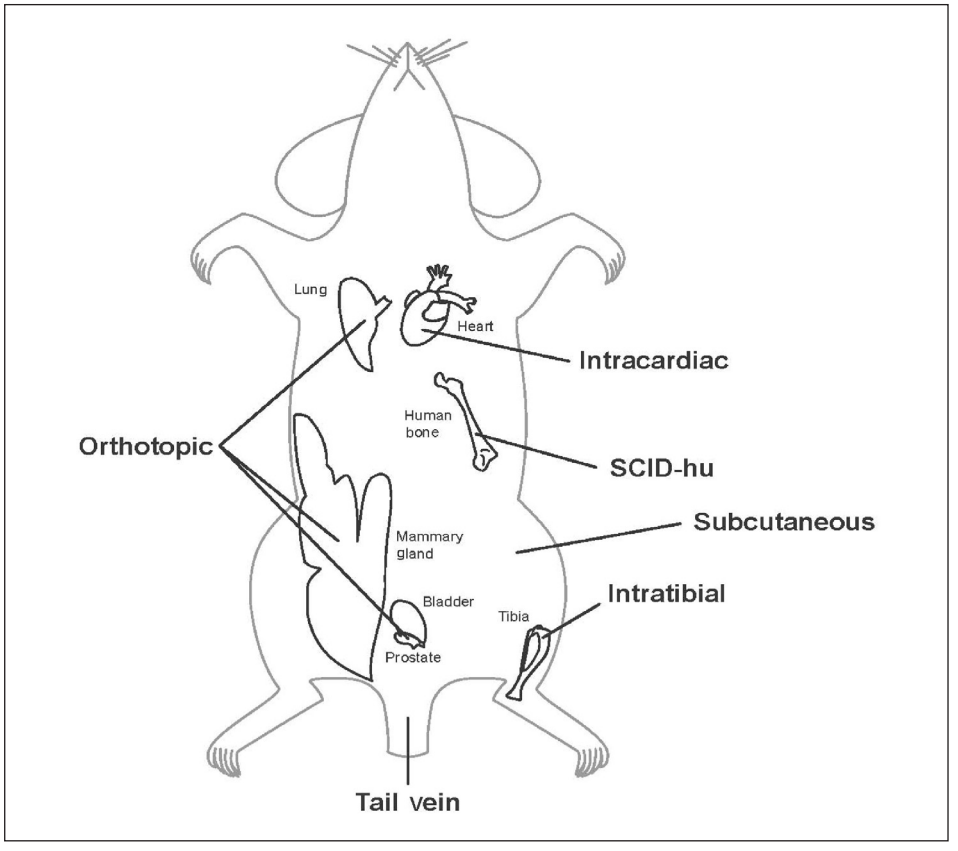

Figure 3. **Experimental models of skeletal metastasis.** Tumor cells can be injected at any of the sites shown to generate a metastasis model. Note that the intracardiac, intravenous, and intratibial models can only establish models of experimental metastasis that do not reflect the entire process of metastasis (see the description of the process of metastasis in the "Skeletal Metastasis" section of this review). However, spontaneous metastasis can be achieved by the orthotopic and subcutaneous models, which generate primary tumors prior to metastasizing.

of injection, it provides valuable information as to how cancer cells go through the entire process of primary tumor formation through the growth and maintenance of a metastatic lesion. Cancer cells injected into the organ of their origin theoretically behave as they would in the original setting because the microenvironment of the primary tumor host organ is thought to be important for the development of metastasis[164]. However, the microenvironments of the human and the mouse differ, which may explain why so few bone metastases occur in these mouse models.

Another important use of orthotopic models is to select cell lines that are more metastatic or more likely to metastasize to particular organs[165]. For instance, the C4-2 prostate cancer cell lines were derived from the LNCaP cell line that was orthotopically and subcutaneously injected into nude mice. After the mice were castrated and metastasis occurred, metastatic cells were removed from lymph nodes or the bone and immortalized into novel cell lines, called C4-2B (derived from bone metastasis) and C4-2Ln (derived from lymph node metastasis)[166,167]. C4-2B cells are now commonly used to induce prostate cancer skeletal metastasis in research into the efficacy of novel therapeutics.

Intraosseous injections

Intraosseous injections are generally done in the tibia or femur of the mouse. This model is not a literal metastatic model because the cells do not need to metastasize from the primary tumor to a distant site. However, this model can provide information about how cancer cells grow and maintain tumors in the bone, as well as about the interactions of cancer cells with the bone microenvironment. It is easier to induce tumor growth in the bone using intraosseous injections than any other type of injection[168].

Because of the bone remodeling that takes place in the multitude of mouse models, the pathology of the bones in these models must be extensively characterized. Assays can be conducted to ascertain the osteoblastic or osteoclastic nature of the bones and the lesions in them. Radiographic imaging is commonly used to evaluate the local bone mineral density or bone morphologic changes[136]. Histological tartrate resistant acid phosphatase (TRAP) staining is used to look into the *in vivo* osteoclastogenesis induced by cancer cells, and it can also be used for *in vitro* co-culture assays. Alkaline phosphatase staining can be used to test osteoblast activity, and Von Kossa staining or alizarin red staining can be used to assess bone mineralization[112]. In addition, single-photon emission computed tomography (SPECT) is a valuable imaging tool to determine where bone turnover is occurring, and it can be used to calculate specific bone parameters and their changes.

Intracardiac, intravenous, and subcutaneous injections

Compared with direct intraosseous injection (which is mainly for studying tumor-bone interactions), intracardiac, intravenous, or subcutaneous injections better mimic spontaneous bone metastasis in humans. In these models, metastases most commonly form in the lungs because of the direct injection of cells into the blood stream[169], avoiding the steps of invasion and intravasation of tumor cells. These types of injections allow us to study the types of homing that occur during metastasis and to examine the interaction of tumor cells with the microenvironment of the tissue being invaded.

SCID-human models

One model that could incorporate any type of injection described above is the implantation of human adult or fetal bone together with subcutaneous injection of cancer cells into immune-deficient mice[170-172]. This model is commonly referred to the SCID-hu model. This model provides a human bone microenvironment on which cancer cells can home, providing a more accurate representation of human skeletal metastasis. In several experiments, the human cancer cells preferentially homed to the human bone over mouse bone[173]. A possible negative to this model is that the fetal bone is mostly woven bone, whereas adult bone consists of mature lamellar bone. If adult human bone is used, there are many biological variables arising from the source of the bone that could complicate the interpretation of results.

Summary

No current experimental model accurately recapitulates bone metastasis, so researchers often choose a model having a specific characteristic relative to the system they are studying. It is unlikely that there will ever be a perfect mouse model of skeletal metastasis, due to the heterogeneous nature of cancer and metastasis, envi-ronmental factors that cannot be mimicked, and the fact that human and mouse genetics and physiology are not identical. Today, however, the mouse model is the mainstay of experimentation in the skeletal metastasis field. There are several excellent reviews that provide additional information on mouse models and their role in skeletal metastasis research[174-176].

Conclusion

Metastasis and growth of cancer cells in the bone is a complex problem with no reliable cure. The current clinical treatments do little to prolong the life

span of patients. Future treatments will likely target not only the primary tumor but also components of the microenvironment surrounding tumor lesions. The solution likely lies in the synthesis of ideas and technologies from several fields. Because of the role of the Wnt signaling in carcinogenesis and metastasis, the potential for Wnt antagonists as either primary or adjuvant therapeutics is an area of promising study. In humans, aberrant Wnt activation is seen in primary tumors, and increases in osteoblastic metastatic bone lesions. In mice, the Wnt signaling can induce cancer when activated in epithelial cells. So far, it appears that activated Wnt signaling in mice causes carcinoma *in situ*, but no metastasis has been found in these models. A significant knowledge gap in the field is the reason that activating Wnt in the mouse does not result in metastatic cancer. Several potential explanations exist for this. The Wnt signaling may be a second genetic event that pushes primary tumors toward a metastatic phenotype. In prostate cancer, Wnt activation may increase in response to decreasing levels of circulating androgens during androgen deprivation therapy and then physically bind to the androgen receptor to induce castration-resistant prostate cancer. It is possible that these differences are due to the species-specific differences. However, it is likely that mouse models will continue to provide valuable information for these studies. Improvement in the ability of these models to accurately replicate key aspects of human prostate tumorigenesis will allow assessment of these difficult questions and provide the foundation for the development of improved treatment of cancer patients with skeletal metastasis.

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