Emerging and Established Models of Bone Metastasis

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1. Introduction

Bone is a common site of metastatic cancer, with an estimated 280,000 adults in the United States suffering from metastatic bone disease.[1] The cancers that most commonly metastasize to bone are prostate and breast cancer, which are also two of the most common cancers in the United States.[2-4] Additionally, lung, thyroid, and kidney primary tumors are reported to metastasize to bone, albeit less frequently.[2] These bone lesions cause serious skeletal complications, including spinal cord or nerve root compression, hypercalcemia of malignancy, pathologic fractures, and debilitating bone pain.[1] Furthermore, the median survival after a diagnosis of overt skeletal metastases is approximately 2-3 years.[4,5] These aforementioned facts illustrate the clinical importance of preventing or curing bone metastasis. Despite this, current treatment options for patients with bone metastases are seldom curative, and are instead mostly palliative.[2] Further, metastatic bone disease poses a significant burden on the healthcare economy. Accordingly, Schulman et al. [6] estimated care for patients with bone metastases cost the United States thirteen billion dollars in 2005 alone. With the current emphasis on decreasing healthcare expenditure, a significant step towards a curative and/or preventive treatment for bone metastases would undoubtedly address a clinical and economic problem in one fell swoop.

The largest barrier to clinical translation in bone metastasis research is the lack of an appropriate in vivo animal model.[7-9] This lack is due to several factors, the most glaring being our incomplete...
understanding of the complex pathophysiological mechanisms at play during bone metastasis.[2,8] Increased knowledge of cancer cell osteotropism would be the foundation for the development of a more curative type of care. Therefore, the purpose of this review is to evaluate the current bone metastasis models and identify future directions for improvement.

2. Biology of Bone Metastasis

Stephen Paget first described a non-random pattern of metastasis to organs in 1889 while analyzing autopsy specimens of women who had died of breast cancer.[10] Paget developed the “seed and soil” hypothesis which compared disseminated cancer cells to seeds being dispersed, while noting that plants will only grow if the seeds end up in congenial soil. In this example osteotropic cells are the seeds and the bone/bone marrow microenvironment acts as a fertile soil for them to grow. Since the advent of the “seed and soil” hypothesis our understanding of metastatic mechanisms has significantly increased; however, this remains the backbone of the basic concept of cancer cell homing during bone metastasis.

Tumor metastasis is a multistep process consisting of tumor growth, angiogenesis, intravasation, survival in the circulation, and extravasation.[4] Tumors shed approximately 3.2 x 10^6 cells/g tissue per day; however only 0.01% of these cells survive the rigors of the systemic circulation and develop into metastases.[11,12] Furthermore, shed circulating tumor cells are predicted to comprise 1 cell out of 10^5-10^7 leukocytes in the bloodstream.[13] The cells that metastasize escape the primary tumor by releasing proteases allowing them to cross the endothelium of small blood vessels, entering the circulation, and homing to distant organs, including bone.[2] Bone is a common site of metastasis due to the high blood flow in the red marrow, presence of adhesive cells, mechanical support, and production of angiogenic and bone-resorbing factors that enhance tumor growth.[2,9] However, which factors control the homing of circulating tumor cells to the bone remain to be discovered. Once cancer cells have survived the rigors of the systemic circulation, they invade the bone marrow and must possess certain phenotypic characteristics for overt bone metastasis to occur.[2] To colonize the bone, tumors cells must migrate across the sinusoidal wall which allows them to co-opt the hematopoietic stem cell (HSC) niche of the bone marrow. In doing so, these cancer cells compete in the surrounding tissue and cause HSC to evacuate the bone marrow. In addition, the cancer cells acquire the HSC’s mechanisms of proliferation and chemotaxis in which they previously used for blood cell production.[14] One way tumors cells home to an colonize bone is via the CXCL12/CXCR4 signaling axis. Receptor CXCR4 on cancer cells at the primary tumor site responds to CXCL12/Stromal derived factor-1α, which is secreted into circulation by osteoblasts, inducing chemotaxis and further homing to and accumulation in the bone. The disseminated tumor cells must then survive, stimulate angiogenesis, and migrate to the bone surface. The tumor cells release signaling proteins, such as vascular endothelial growth factor (VEGF), parathyroid hormone-related peptide (PTH-rp), bone morphogenic protein (BMP), and wingless (WNT), that stimulate the displacement of osteoblasts lining the bone surface, activating bone resorption by osteoclasts, and allowing tumor cell infiltration of the surface of the demineralized bone.[15] However, the micro-processes that regulate the cancer cells movement and survival upon arrival at the distant organ remain elusive.[4] In both advanced breast and prostate cancer, there is about a 70% chance of their primary cancers metastasizing to bone. However, for prostate cancer patients in particular, most patients will die from other causes before overt bone metastases occur. This is due to the tendency of disseminate tumors cells to initially become dormant after colonizing the bone.

2.1 Dormant Lesions

One of the most perplexing mysteries surrounding metastatic disease is the concept of dormancy.[16] This is a phenomenon where disseminated tumor cells persist in a long term state of quiescence and are eventually re-activated to induce metastatic relapse.[17] This can occur months to years after resolution of the primary tumor, with tumor cells remaining dormant within the bone.
The presence of disseminated tumor cells in a patient with no evidence of disease puts the patient at a higher risk for relapse. Metastatic dormancy has remained understudied in part due to the lack of appropriate animal models to do so. Once cancer cells are reactivated, lesions can either be osteolytic (bone destructive), osteoblastic (bone forming), or mixed. Breast cancer commonly results in an osteolytic metastasis (73%) while prostate cancer results in an osteoblastic metastasis (68%). Other advanced cancers (lung, melanoma, thyroid, kidney, and gastrointestinal) have demonstrated bone metastasis; however, not with the same frequency.

2.2 Osteolytic Lesions

Osteolytic lesions are caused by overactivation of bone resorption. Disseminated tumor cells initiating metastatic lesions enter the bone surface by stimulating osteolysis via enhanced osteoclast differentiation. Osteoclasts originate from hematopoietic precursor cells in the bone marrow and have a primary role of bone resorption. Continued stimulation and loss of bone resorption regulation by osteoclast activation forms the basis of an osteolytic lesion (Figure 1a). The most established growth factor in bone that contributes to osteolytic lesions is transforming growth factor-beta (TGF-β). It is theorized that TGF-β induces pro-osteolytic gene expression with PTH-rp release proliferation. This increases osteoblastic production of receptor activator of nuclear factor-kappa B (RANK) ligand, therefore, indirectly stimulating osteoclast formation (Figure 1a). Cancer cells themselves can also produce RANKL increasing osteoclast activation. Continued bone resorption causes release of more bone matrix proteins and growth factors that stimulate further tumor cell proliferation, leading to a cruel cycle of osteolysis. Furthermore, TGF-β increases cyclooxygenase-2 expression, which correlates with an increase in interleukin (IL)-8. IL-8 induces osteoclast formation and activity independent of the RANK-ligand pathway. This continued breakdown of the bone structure contributes to the bone pain and pathological fractures experienced by patients with osteolytic bone metastases.

2.3 Osteoblastic Lesions

Osteoblastic lesions are characterized by increased bone formation. Metastatic lesions from prostate carcinomas are the most well-known producer of osteoblastic lesions. Osteoblasts originate from mesenchymal progenitor cells and function by forming bone. They do so by the stages of proliferation, matrix maturation, and mineralization. Growth of prostate cancer cells alters bone remodeling by secreting factors that directly affect the osteoblast and osteoclast relationship (Figure 1b). The cancer
cells produce RANK ligand and osteoprotegerin (OPG), thereby disrupting the balance in normal osteoclast activity.\[29\] Furthermore, there is an abundant release of TGF-β and vascular endothelial growth factor (VEGF) by the cancer cells, which directly affect the osteoblast activity.\[30\] This is done through the WNT pathway, which is implicated in osteoblastogenesis.\[20,31\] The combination of this WNT pathway upregulation coupled with the reported decreased expression of the WNT antagonist, dkkopf-1, in patients with advanced prostate cancer is associated with the formation of osteoblastic lesions (Figure 2).\[32\] Finally, the prostate cancer cells have been shown to express large amounts of factors that strengthen the osteomimicry.\[33\] There is some evidence that distant tumors induce osteoblast activation and bone formation prior to metastasis occurring as part of preparation of the premetastatic niche.\[34\] While areas of increased bone may seem beneficial, the inconsistent structure that results leads to unequal distribution of mechanical loads through the bone producing bone fractures. In many patients, mixed lesions of osteolytic and osteoblastic sites increase the risk of fractures and the structure of the bone becomes even more patchworked. How each type of lesions is initiated and progresses remains a mystery which will eventually be solved through new bone metastasis models.

3. In Vivo Models of Bone Metastasis

Our lack of understanding regarding bone metastasis stems directly from the fact that there are currently no suitable animal models to mimic human tumor cell metastasis to the bone microenvironment. The importance of in vivo studies in developing new therapeutic methods to combat the effects of metastatic disease cannot be understated. Prior to embarking on clinical trials in human patients a new therapy must first be thoroughly tested in animal models.\[35\] However; the animal model used should reflect the environment that will be encountered in the human body. There are currently several in vivo models that exist to evaluate bone metastases; however, they all have their limitations.\[9,36\]

3.1 Spontaneous Bone Metastasis

Spontaneous bone metastasis in animal models are currently non-existent because this phenomenon is rare and difficult to recreate in most animal species.\[36-38\] However, a select few reports of metastatic disease in large animals (canine and feline) to bone have been reported.\[38\]. There is a single report of lung adenocarcinoma in a feline species that underwent spontaneous metastasis to bone.\[39\] However; this is rare and does not present a feasible avenue for future research modeling. Canines are the only animal where prostatic cancers metastasize to bone reliably due to canine prostatic tissue undergoing similar changes to humans.\[38\] Despite this, the rarity and difficult identification does not allow suitable models to reliably be recreated.\[37,38\] Further due to the small numbers of animals per arm and the cost of care render large animal models particularly unsuitable for initial testing of treatments. Thus, additional models were developed in rodents but these models do not mimic the process of spontaneous metastasis. In the few rodents and larger animals in which spontaneous does occur, the progression is slow requiring months or years of tracking the animals and the timeline is prohibitive for testing therapeutic interventions. Thus, the field has focused on developing models of bone metastasis that will progress quickly and occur reliably in most animals.

3.2 Orthotopic and Intracardiac Models

Another method of investigating the biological progression of metastatic bone lesions involves primary colonization of the bone with cancer cells. Injection of cells into the tibia or femur of a mouse is termed an orthotopic model and allows incorporation of the cells that can replicate tumor-induced changes in murine bone.\[40-42\] A series of orthotopic models are listed in Table 1. Direction injection into the bone microenvironment results in overt metastasis arising quickly allowing for treatments to be tested for slowing or preventing metastatic growth. The limitation to this model is that it only resembles
the final stages of bone colonization preventing the study of homing, extravasation and dormancy, and thus is more analogous to a primary tumor model.\[9\]

### Table 1. Orthotopic Models

<table>
<thead>
<tr>
<th>Study</th>
<th>Cell Line Used</th>
<th>Cancer Type</th>
<th>Animal Used</th>
<th>Methodology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ooi et al. [38]</td>
<td>MCF-7</td>
<td>Breast</td>
<td>Nude mice</td>
<td>Injected into anterior tuberosity of proximal tibia in both limbs</td>
</tr>
<tr>
<td>Le Gall et al. [39]</td>
<td>BT474</td>
<td>Breast</td>
<td>Nude mice</td>
<td>B02 cells were injected into the tail vein after BT474 cells were inoculated in the bone marrow</td>
</tr>
<tr>
<td>Zheng et al. [40]</td>
<td>MCF-7</td>
<td>Breast</td>
<td>Nude mice</td>
<td>Cells injected into tibial marrow canal</td>
</tr>
</tbody>
</table>

To solve this problem and create a more metastatic model, some groups attempted intracardiac injection of osteotropic cancer cells, to quickly induce bone metastasis at a high frequency.\[43-46\] Some current intracardiac injection models are listed in Table 2. In addition, tail vein injections are performed to mimic hematogenous metastasis. These models recapitulated extravasation and colonization and the cells may undergo dormancy during the metastatic progression. Many of these models rely on human cell lines to study osteotropism. The use of a xenograft presented a major limitation in that to avoid graft rejection, immune compromised hosts are necessary. This eliminates the ability to examine the role of the immune system in tumor progression.

### Table 2. Intracardiac Models

<table>
<thead>
<tr>
<th>Study</th>
<th>Cell Line Used</th>
<th>Cancer Type</th>
<th>Animal Used</th>
<th>Methodology and Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yodena et al. [41]</td>
<td>MDA-MB-231</td>
<td>Breast</td>
<td>Nude Mice</td>
<td>Spread was mostly to bone, but occasionally to adrenal glands, ovary, and brain 3-4 weeks after inoculation.</td>
</tr>
<tr>
<td>Henriksen et al. [42]</td>
<td>MT-1</td>
<td>Breast</td>
<td>Nude rats</td>
<td>N/A</td>
</tr>
<tr>
<td>Yi et al. [43]</td>
<td>MCF-7</td>
<td>Breast</td>
<td>Nude mice</td>
<td>N/A</td>
</tr>
<tr>
<td>Canon et al. [44]</td>
<td>MDA-MB-231</td>
<td>Breast</td>
<td>Nude mice</td>
<td>Cells were luciferase labelled</td>
</tr>
</tbody>
</table>

### 3.3 Immunocompetent Models

Due to the known link between the immune system and the skeletal system in cellular mechanisms, the science of “osteoimmunology” began to gain attention.\[47,48\] Osteoimmunology references the link discovered between T-cell activation and bone resorption, particularly that seen with metastatic bone lesions.\[49\] The skeletal and immune systems share regulatory molecules; thus, disseminated tumor cells that act on the skeleton may also have an effect on the immune system, or vice versa.\[49\] Therefore bone metastasis models were developed using immunocompetent mice for murine breast cancer, melanoma, and prostate cancer cell lines to investigate any effects the immune system may have (Table 3).\[50-52\] These models represent a tremendous advancement in pre-clinical models of bone metastasis; however, most still require an intracardiac injection of cancer cells. Although this is a reproducible technique, it would lead to obvious systemic issues that may affect the mechanisms being investigated within the bone. Furthermore, this has limited translational applicability due to differences in species related differences.\[8\] Most immunocompetent models require the injection of cells directly into the circulation and are not models of spontaneous metastasis. The models are useful in examining homing and colonization but lack the ability to study intravasation and premetastatic niche formation due to the lack of a primary tumor.
Table 3. Immunocompetent Models

<table>
<thead>
<tr>
<th>Study</th>
<th>Cell Line Used</th>
<th>Cancer Type</th>
<th>Animal Used</th>
<th>Methodology and Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Power et al. [48]</td>
<td>RM1</td>
<td>Prostate</td>
<td>C57Bl/6 mice</td>
<td>Demonstrated no preference for particular bone sites</td>
</tr>
<tr>
<td>Ruttinger et al. [49]</td>
<td>P2 and 4T1</td>
<td>Melanoma and Breast</td>
<td>C57Bl/6 and BALB/c mice</td>
<td>Studied tumor regression with anti-CD3 activated and IL-2 expanded tumor vaccine</td>
</tr>
<tr>
<td>Arguello et al. [50]</td>
<td>B16</td>
<td>Melanoma</td>
<td>C57Bl/6 mice</td>
<td>Injection sites include left ventricle and mouse tail vein</td>
</tr>
</tbody>
</table>

3.4 Humanized and Tissue-Engineered Models

Another alternative model that exists and has grown in popularity is the use of a “humanized” model for metastasis.[8] The aim of these models is to use human cancer cells and a human bone implant to serve as the target for metastasis.[53-58] A list of humanized models can be found in Table 4. Humanized models attempt to recapitulate the human immune system in mice to better represent progression towards metastasis in patients. In these models often still use directing injection of tumor cells into the circulation but newer models may involve spontaneous metastasis from a primary tumor. However, the availability of human tissues is limited and therefore several authors have implemented tissue engineering in order to create a reproducible and controllable microenvironment.[9,59,60]

Table 4. Humanized Models

<table>
<thead>
<tr>
<th>Study</th>
<th>Cell Line Used</th>
<th>Cancer Type</th>
<th>Animal Used</th>
<th>Scaffold Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nemeth et al. [51]</td>
<td>DU145, LNCaP, and PC3</td>
<td>Prostate</td>
<td>SCID-hu mice</td>
<td>Human fetal human bone fragments</td>
</tr>
<tr>
<td>Yonou et al. [52]</td>
<td>LNCaP and PC3</td>
<td>Prostate</td>
<td>NOD/SCID mice</td>
<td>Human adult cancellous rib fragments from lung cancer patients</td>
</tr>
<tr>
<td>Kuperwasser et al. [53]</td>
<td>SUM1315</td>
<td>Breast</td>
<td>NOD/SCID mice</td>
<td>Human bone used from discarded femoral heads from patients undergoing total hip replacement</td>
</tr>
<tr>
<td>Yang et al. [54]</td>
<td>GFP-MDA-MB-231</td>
<td>Breast</td>
<td>NOD/SCID mice</td>
<td>Morselized human bone implants</td>
</tr>
<tr>
<td>Xia et al. [55]</td>
<td>SUM1315</td>
<td>Breast</td>
<td>NOD/SCID-hu mice</td>
<td>Female human bone tissues were obtained from discarded femoral heads from patients undergoing total hip replacement</td>
</tr>
</tbody>
</table>

Tissue-engineered bone metastasis models, listed in Table 5, take advantage of recent advances in regenerative medicine to create a new bone microenvironment using scaffolds. The various scaffold materials provide structural support and environmental cues promoting osteoblast differentiation and function. Depending on the cells used to seed scaffolds, the entire heterogeneity of the bone marrow may or may not be represented. Nevertheless, current models incorporating this technique still rely upon an
intracardiac injection and immunocompromised animals, and therefore will be subject to systemic issues and a lack of immune response, as discussed previously.[8]

### Table 5. Tissue Engineered Models

<table>
<thead>
<tr>
<th>Study</th>
<th>Cell Line Used</th>
<th>Cancer Type</th>
<th>Animal Used</th>
<th>Scaffolds and Methodology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moreau et al.</td>
<td>SUM1315</td>
<td>Breast</td>
<td>NOD/SCID mice</td>
<td>Silk fibrin scaffolds coupled with BMP-2 and human bone marrow stromal cells were used</td>
</tr>
<tr>
<td>Schuster et al.</td>
<td>PC3 and H460</td>
<td>Prostate and Lung</td>
<td>SCID mice</td>
<td>Mature osteoblasts were loaded on hydroxyapatite-coated collagen sponges</td>
</tr>
<tr>
<td>Thibaudeau et al. [9]</td>
<td>MDA-MB-231</td>
<td>Breast</td>
<td>NOD/SCID mice</td>
<td>Human osteoblast cell-seeded melt electrospun polycaprolactone scaffolds + recombinant human BMP-7</td>
</tr>
</tbody>
</table>

#### 3.5 In vivo Dormancy Models

One final limitation to current in vivo bone metastasis models revolves around the inability to recapitulate dormancy and homing.[17,18] Xenograft models have provided the minimal knowledge garnered on homing and dormancy. The basis of these models is that cell cycle arrest of cancer cells can be controlled and is reversible by either a change in microenvironment or by inhibiting signaling pathways.[61-63] There appears to be one attempt in the literature to incorporate dormancy into an in vivo models; however, this has only reliably recreated dormancy in some of the breast cancer lines investigated.[61] The authors used 3D biomatrices containing bone marrow stem cells and breast cancer cells (MDA-MB-231) and subcutaneously implanted these into NOD/SCID mice. After 24 hours either a supportive (DMSO) or inhibitory niche (activating receptor-like kinase inhibitors - SB431542, SB203580, and S1042) seeded 3D biomatrix was implanted on the contralateral side, and tumors grew within a supportive niche, but no tumors found in the inhibitory niche. The authors demonstrated that cancer cells at the original seeding density were present within the inhibitory site, thus proving that the cancer cells did not proliferate nor die; therefore, concluded that the remaining cancer cells were dormant. However, due to the paucity in research in this area there is vast room for growth in the future.

#### 4. Future Directions

Despite the push towards a focus on in vivo models by some, others believe that the ideal way to investigate the complex molecular mechanisms involved in this process is by advanced in vitro modeling.[64-70] These models consist of microfluidic models or advanced mathematical modeling among others.

#### 4.1 Microfluidic Models of Metastasis

The general principle behind a microfluidic model is to recreate the 3-dimensional (3D) microenvironment of in vivo tissues, while also allowing the researcher to have complete control of the microenvironment.[65] This allows for metastatic migration from a 3D origin tissue to a 3D target tissue, within a controllable fluidic environment.[65] Four models for bone metastasis in a microfluidic model have been identified in the literature.[67,71,72] Bersini and Jeong [71,72] used a tri-culture system, consisting of osteo-differentiated human bone marrow (h-BM) mesenchymal stem cells (MCSs), endothelial cell monolayer, and human breast cancer cells (MDA-MB-231). With this model the authors demonstrated that breast cancer cells extravasated into the bone microenvironment significantly more than a collagen control, and that this increase in extravasation was associated with cross-talk between
the h-BM MSCs and the MDA-MB231 cells through CXCL5-CXCR2 paracrine signaling pathways.[71] The authors then refined this system by introducing human umbilical vein endothelial cells into the initial culture of the bone micro-environment in order to induce a microvascularized bone environment.[72] This allowed the authors to identify that the breast cancer cells responded to bone stromal cells through aforementioned paracrine signaling, again leading to extravasation. Through the use of this novel model, the authors also identified that the myoblast cell line C2C12 had a protective effect against metastasis. Finally, the most recent microfluidic model to be introduced is from Hau et al.[67] The authors attempted to identify weak areas in the model presented by Jeong and Bersini and the main limitation to improve upon was to allow maturation and growth of the osteoblastic cell lineage, allowing mineralization and natural collagen fiber organization that may be involved in the complex underlying metastatic mechanisms. This was performed by using a miniaturized bone on a chip model consisting of two compartments. The first of these allows for medium changes, while the second allows for osteoblastic tissue growth. The authors used MC3T3-E1 bone cells in a miniaturized bone-on-chip model with resultant spontaneous formation of thick, mineralized osteoblastic tissue. Furthermore, their co-culture with MDA-MB-231 and osteoblastic tissues demonstrated trademarks of breast cancer colonization. While these microfluidic models lack some complexity of the in vivo models, including a functional immune system, they are ideal for high throughput screening of potential therapeutic aimed at preventing or slowing metastasis.

4.2 In Silico Models of Metastasis

Another method to identify potential therapeutic targets for metastasis is through advanced computational modeling allowing for the integration of key biological findings with the power of advanced computational measurements and calculations.[64] This method permits the study of the numerous cellular effects and molecular interactions simultaneously and is beginning to increase in popularity.[64,73-77] Araujo et al.[64] developed a model that considered osteoblasts (MC3T3), osteoclasts, precursor osteoblasts, precursor osteoclasts, MSCs, and prostate cancer cells. The authors demonstrated that MSC recruitment is a vital step in formation of metastatic lesions and that the growth rate calculated using this model was comparable to in vivo experiments, therefore, outlining the utility of their computational model. Computational models, such as this one, are becoming more common with advancing technologies. It is our opinion that use of these models may surpass those of in vivo and classic in vitro models in the future; however, this appears to still be in the early stages.

5. Conclusion

Significant progress has been made in the regeneration of a metastatic bone environment in an in vivo environment, but several barriers still exist. The major barriers include the use of intracardiac injections and the use of immunocompromised animals. The adaptation of using tissue engineered constructs may eventually lead to the ideal model. Future research should focus on using non-reactive tissue engineered implants to create a humanized environment, without invoking a host immune response. Furthermore, the ability to inject cancer cells of choice in more of an anatomic position (e.g. the mammary fat pad for breast cancer) would allow for creation of a more translatable in vivo model.

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