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## 3d Tissue Engineered In Vitro Models Of Cancer In Bone

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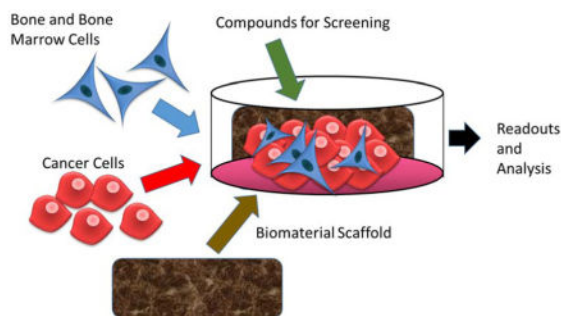
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### Abstract

Biological models are necessary tools for gaining insight into underlying mechanisms governing complex pathologies such as cancer in the bone. Models range from *in vitro* tissue culture systems to *in vivo* models and can be used with corresponding epidemiological and clinical data to understand disease etiology, progression, driver mutations, and signaling pathways. In bone cancer, as with many other cancers, *in vivo* models are often too complex to study specific cell-cell interactions or protein roles, and 2D models are often too simple to accurately represent disease processes. Consequently, researchers have increasingly turned to 3D *in vitro* tissue engineered models as a useful compromise. In this review, tissue engineered 3D models of bone and cancer are described in depth and compared to 2D models. Biomaterials and cell types used are described, and future directions in the field of tissue engineered bone cancer models are proposed.

### Graphical Abstract



### Keywords

Bone; Bone Marrow; Bone Marrow Adipose Tissue; Osteotropism; Breast Cancer Bone Metastasis; Biomaterials; Silk; Scaffolds

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## Introduction

The goal of using disease models for bone cancer, or cancers that spread to bone, is to gain maximal information about how cancer and bone cells interact with each other. The accuracy of these models is crucial for researchers to determine how these interactions support cancer growth and lead to cancer-induced bone disease (e.g., osteolysis, as found in myeloma and metastatic breast cancer, or osteoblastic disease, as seen in prostate cancer metastasis). Tissue engineered disease models allow us to identify novel molecular targets, drug delivery mechanisms and disease biomarkers, which leads to better therapeutics or diagnostics for translation into the clinical setting. Cancer models in general are especially essential as cancer is the second leading cause of death in the United States and is responsible for nearly 1 of every 4 deaths.<sup>1</sup> According to the American Cancer Society, about 1 million new cancer cases were diagnosed in 2016, and about 595,690 Americans died of cancer in 2016.<sup>2</sup> However, cancer death rates have decreased by 23% between 1991 and 2012. This can be attributed to better screening and earlier diagnoses as well as the development of novel cancer therapies, supported by improved preclinical cancer research.<sup>2</sup>

Breast and prostate cancer, the most common cancers in women and men respectively,<sup>2</sup> are fatal due to their highly metastatic potential during late/advanced disease stage. Commonly these cancers metastasize to the bone, wreaking havoc on the endocrine system, skeletal mechanical stability, hematopoiesis, and immune function. Metastasis is responsible for 90% of cancer mortality with solid tumors.<sup>2</sup> Bone is one of the most common sites of metastasis in breast, prostate, and lung cancer. Bone is also frequently the site of metastasis in other cancers such as thyroid cancer, renal cancer, lymphoma, and Ewing's sarcoma.<sup>2</sup> Multiple myeloma not only initiates in the bone marrow (BM), but also disseminates from its original location and spreads throughout the BM; thus myeloma can also be thought of as a bone-metastatic cancer. Myeloma and other bone-metastatic cancer cells interact with BM cells that support their proliferation, chemoresistance, and evolution. Understanding the roles of these bone cells (osteoblasts, osteocytes, osteoclasts, hematopoietic cells, mesenchymal cells, and immune cells) and how they network and interact with tumor cells has proven useful in developing therapies for primary bone cancer and metastases. This phenomenon of microenvironmental support of tumors falls in line with Dr. Paget's "seed and soil" hypothesis in which neoplastic cells (or "seeds") only grow in hospitable bone marrow (a suitable "soil").<sup>3</sup> Making the soil less suitable for tumor cell homing or colonization inhibits tumor cell proliferation and disease progression. BM is rich in chemokines and growth factors, contains unique immune and stromal cell composition and has fenestrated blood vessels expressing specific molecules that uniquely facilitate tumor homing.<sup>4-11</sup> In the bone and BM, tumor cells can influence mesenchymal and hematopoietic progenitors and mature cells, which can contribute to disease progression in many ways.<sup>1</sup> Osteolytic tumors typically induce a vicious cycle by inhibiting osteoblastic activity while stimulating osteoclastic activity.<sup>12</sup> Osteoblastic tumors typically do the opposite; these stimulate osteoblasts to rapidly produce excess bone matrix, leading to mechanically weak and dysfunctional bone matrix. Bone metastasis is especially insidious because tumor cells can lie dormant for a variable amount of time (from months to years) before becoming activated.

<sup>3</sup> Currently there is a need for a better understanding of the molecular pathways that govern bone metastasis to lead to better therapies and clinical outcomes for patients.

The standard method for cancer research is 2D cell culture, animal modeling, and subsequently clinical trials. More research into the efficiency of this pipeline has demonstrated that many findings in 2D culture do not translate to patients, in part due to differences between *in vitro* and *in vivo* culture as well as differences between 2D and 3D culture. Numerous researchers have found that gene expression and cell function in 2D cell culture do not closely mimic physiological gene expression found *in vivo* and many normal and malignant cells sense and respond to 3D environments differently than 2D environments.<sup>5</sup>

In the past few decades, a plethora of novel tissue engineered disease models of cancer have been developed through optimization of the biomaterials, bioreactors, and culture conditions for 3D culture.<sup>13</sup> These models provided the field with new ways to accelerate the rate of cancer research and make clinically relevant discoveries about cancer biology by decreasing time wasted in unsuccessful *in vivo* models or failed clinical trials (Figure 1). This review discusses the numerous approaches in developing models to interrogate bone metastasis and growth in terms of cell-to-cell interactions, molecular pathway signaling, gene expression, and metabolism. Overall, this review provides a synopsis of the different types of biomaterials that are used and their relative advantages and disadvantages in the current undertaking to recapitulate the bone tumor microenvironment as accurately as possible. It concludes with some recommendations and potential future directions.

### 3D Models

Using 3D cell culture to recapitulate the tumor bone microenvironment provides an in-depth understanding of bone metastasis and cancer pathogenesis by more accurately modeling dynamic cell-cell and cell-ECM interactions. However, there are still many avenues to explore to optimize 3D cell culture by finding more suitable biomaterials for models in terms of cellular growth, reproducibility, scalability, and cost. When choosing a biomaterial for a 3D model, there are many properties to consider including longevity, biocompatibility, method of degradation, mechanics (strength, elasticity/plasticity, termed “viscoelasticity”), porosity, molecular gradients and mass transport (access to oxygen, nutrients and various soluble factors), optics, adhesion or signaling sites, surface roughness, shape, type (e.g., sponge or hydrogel), and source (natural vs. synthetic).<sup>12,14–16</sup> Synthetic materials may allow for more control, but natural materials often promote more native cell-cell and cell-matrix signaling. The correct biomaterial must correctly regulate cellular behavior to more realistically recapitulate the biochemical signals, physiological environment, and topographical cues seen *in vivo*. The scaffold must provide the correct mechanical and functional environmental signals (e.g., surface chemistry and architecture)<sup>17</sup> while encouraging cell adhesion, proliferation, and migration without evoking an inflammatory or apoptotic response. These properties are critical in recreating distinct tumor niches that are reproducible and relevant to *in vivo* physiology. Table 1 summarizes the biomaterials currently used for 3D bone cancer models.

## Scaffold-Based Modeling Techniques

Scaffolds provide robust support for cell growth and they can simulate native extracellular matrix (ECM) in many of their properties. They can be made from natural sources such as collagen, Matrigel, silk, and agarose, but they can also come from synthetic materials such as poly(ethylene glycol) (PEG), poly(lactic-co-glycolic acid) (PLGA), or a combination of materials.<sup>18</sup> These materials can be used alone or in combination (polyblended) to form different types of scaffolds such as sponges and hydrogels.

**Hydrogels**—Hydrogels are hydrophilic polymer networks that closely mimic properties of soft tissues in their viscoelastic properties and interstitial flow. They can be made of natural polymers (like hyaluronic acid (HA), collagen, silk, alginate, or chitosan), synthetic polymers, or a combination of polymers like chitosan and alginate, or PEG and silk.<sup>19,20</sup> Hydrogels can also be reinforced with fibrous materials, like silk fibers, or microparticles to improve their strength. Hydrogels have been widely used to investigate tumor formation due to their easily tunable biophysical and mechanical properties, ease of synthesis, and reproducibility.<sup>19</sup> For example, HA-based hydrogels have been used to test chemotherapeutics for prostate and endometrial cancers<sup>21</sup> as well as myeloma.<sup>22</sup> Commercially available hydrogels also exist and include Matrigel<sup>5</sup>, Tisseel, Qgel<sup>TM23</sup>, ECMgel, Corgel<sup>TM</sup> BioHydrogel<sup>24</sup>, Bio-Oss<sup>®</sup> Collagen<sup>25</sup>, and Nano Dox<sup>TM26</sup>. Challenges with pre-made hydrogels are their proprietary mixtures and potential lot-to-lot variations.

**Sponges**—Sponges, or sponge-like scaffolds, are stronger and more durable than hydrogel scaffolds and can also be made of one, or more than one, biomaterial. Sponges have a large surface area for cellular attachment and robust mechanical stability enabling long-term culture without disintegration.<sup>12,27</sup> They also provide a comparatively high inter-fiber distance or pore size, compared to hydrogels, which facilitates nutrient and gas exchange as well as cell infiltration, which supports long-term culture.<sup>12,28</sup> These properties make sponges useful for cancer cell culture. Silk sponges have been shown to induce significantly different angiogenic factor expression in osteosarcoma models versus 2D cell culture.<sup>29,30</sup> Sponges with high mechanical stiffness also better recapitulate the ECM of tumors, and they can also be functionalized to stimulate tumor growth to reproduce *in vivo* conditions.<sup>19</sup> Many studies agree that the properties of sponges enable better modeling of cancer-cell-ECM interactions and more accurate screening for cancer cell-drug outcomes.<sup>31</sup>

## Natural Biomaterials

**Silk**—Silk scaffolds are made from fibroin fibers processed from raw silk that can be crafted into various morphologies such as sponges, hydrogels, films, mats, and other shapes, with various degradation profiles based on cross-linking degree and other chemistries.<sup>31</sup> Silk scaffolds are a promising platform for 3D bone modeling due to their strength, toughness, biocompatibility, support for cell adhesion,<sup>32</sup> thermal and chemical stability,<sup>15,31</sup> and porosity.<sup>32</sup> Fibroblasts, osteoblasts, hepatocytes, neurons, macrophages, keratinocytes, chondrocytes, endothelial cells, and mesenchymal stem cells have all been successfully cultured on silk scaffolds.<sup>15</sup> Silk fibroin can be isolated from *Bombyx mori* or *Antheraea mylitta* silkworms and processed by one of two main methods, aqueous or solvent-based processing, to form silk sponges. The choice of method depends on pore size, degradation

profile, mechanical strength, and surface roughness desired. Correia et al.<sup>33</sup> compared different aqueous methods and a solvent (HFIP) method to each other and also compared these to decellurized bovine trabecular bone, in their research optimizing silk scaffolds for bone formation. Improved osteogenic induction and bone tissue formation were demonstrated by HFIP-based silk scaffold models as compared to the aqueous models. This improvement was confirmed via bone protein production (osteopontin (OPN), collagen type I, and bone sialoprotein), calcium deposition, and total bone volume assays. Architectural changes and mechanical compression from the beginning to the end of culture were also enhanced on HFIP scaffolds, determined by  $\mu$ CT and Young's modulus calculations, due to the deposition of a more robust extracellular matrix.<sup>33</sup> However, the aqueous-based structures were also similar to native bone scaffolds.<sup>33</sup> Scaffold properties of architecture and mechanical stiffness play an important role in how bone formation occurs. The HFIP-derived models demonstrated a higher stiffness than aqueous-based scaffolds thereby providing a better platform for bone formation. Both models provided architecture that altered cellular activity and bone formation, outcomes of which were dependent on pore morphology of the scaffold.<sup>33</sup> As with most biomaterials, methods of manufacturing silk scaffolds are highly dependent on application since different processes yield different mechanical strength and porosities.

Tan et al.<sup>34</sup> used silk scaffolds to show differential gene expression related to chemosensitivity in 2D versus 3D osteosarcoma cultures. Doxorubicin and cisplatin are common therapeutics used in the treatment of osteosarcoma. Doxorubicin is a cell cycle specific agent while cisplatin is a cell cycle non-specific agent. The osteosarcoma cells grown on the 3D scaffold was less resistance to doxorubicin than the 2D model even though their MDR1 (multidrug resistance gene) levels stayed the same. The amount of doxorubicin needed to kill tumors *in vivo* was greater than the 2D cultures could be exposed to without massive cell death. However, when treated with cisplatin, a cell cycle stage non-specific drug, both 3D cell culture and 2D cell culture were similar in sensitivity to the drug. The 3D models exhibited decreased responsivity to doxorubicin similar to clinical *in vivo* responses, unlike 2D models, which implies that drug development using 3D culture would improve drug screening and drug development processes.<sup>34</sup> Gene expression profiles of 3D cultured cells closely reflected mouse xenograft flank tumors and osteosarcoma tumor cells on the scaffold were more resistant to cell cycle specific chemotherapeutics, and the 3D models were much closer to *in vivo* studies in drug resistance than 2D models were.<sup>34</sup> Hence, 3D silk scaffold models are a better comparison to *in vivo* tumor drug resistance than 2D models based on their ability to better approximate osteosarcoma physiology.<sup>34</sup>

Likewise, silk scaffolds have also used to model breast cancer cell metastasis to create targeted therapeutics. Kundu et al.<sup>35</sup> used a silk scaffold-based model for drug testing and screening using drug-loaded folate conjugated nanoparticles with MDA-MB-231 breast cancer cells and MG63 osteoblasts. They explain that, to mitigate the inconsistent and conflicting results of testing drugs in 2D cell culture and to minimize expensive animal testing, the 3D culture was utilized.<sup>35</sup> The targeted, drug-loaded nanoparticles effectively targeted the cancer cells in the 3D model and showed a decrease in cancer cell populations after treatment, while the bone cells remained mostly normal functioning. 3D silk cell culture systems have been used in the investigation of prostate cancer metastasis by Kwon et

al.<sup>36</sup>, as 2D models insufficiently demonstrated information for cellular function and tissue structure and function. Kwon et al.<sup>36</sup> used PC3 prostate cancer cells and 3D silk sponges to create a sustainable 3D tissue system that enabled tumor cells to display an *in vivo*-like behavior, undergoing cell migration and invasion of the engineered bone (silk scaffold) similarly to bone metastasis.<sup>36</sup> They hypothesized that prostate cancer cells have bone cell-like properties that allow them to survive, proliferate, migrate, and invade the bone microenvironment in a process called osteomimicry.<sup>36</sup> This hypothesis was supported by findings from gene expression and cell migration studies on silk scaffolds.<sup>36</sup> The authors' system was a powerful tool for understanding how prostate cancer metastasizes, thereby supporting the development of novel diagnostic methods and therapeutics. Lastly, our lab developed a 3D bone marrow model using silk scaffolds with mechanical properties similar to bone in order to mimic how myeloma cells inhibit osteogenic differentiation.<sup>12</sup> Silk scaffolds supported formation of dense calcified tissue and long-term culture of primary patient myeloma cells.<sup>12</sup> Scaffolds were used to identify microRNAs aberrantly expressed by mesenchymal stem cells (MSCs) co-cultured with myeloma cells, which were found to contribute to the decreased osteoblastic activity seen in myeloma patient bones.<sup>12</sup> We have recently developed a model for 3D bone marrow adipose tissue co-culture with tumor cells, as shown in Figure 2, which allows the researcher the ability to study both the effects of bone marrow adipose on myeloma, and myeloma on bone marrow adipose tissue.

**Collagen**—Another commonly used biomaterial for 3D modeling is collagen, which is biocompatible and biodegradable, and can be engineered and cross-linked to have stronger mechanical properties.<sup>37</sup> Collagen I comprises approximately 95% of the organic matrix of bone and *in vivo* is reinforced with calcium and phosphate ions in the form of hydroxyapatite crystals to give bone its characteristic strength.<sup>37</sup> Herroon et al. explored a novel 3D culture model using collagen to examine the interaction of prostate cancer cells with bone marrow-derived adipocytes, based on the fact that adipocytes in the bone marrow may contribute to tumor growth and metastatic progression of cancers.<sup>38</sup> This team found that 2D cultures did not adequately recapitulate the limited diffusion-driven access to nutrients, growth factors, and signaling molecules in the bone tumor microenvironment.<sup>38</sup> Furthermore, they found that tumor architecture *in vivo* was regulated by 3D spatial confirmation that 2D cell culture did not address.<sup>38</sup> The researchers presented two new 3D models using collagen I gels to investigate bone tumor microenvironment through the interaction of co-cultured BM adipose tissue and metastatic tumor cells.<sup>38</sup>

Tumor cells in the 3D collagen I culture models had a more realistic metabolic response to adipocytes than the 2D culture models, illustrated by immunofluorescence analysis of metabolism-associated proteins, such as carbonic anhydrase 9 and hexokinase 2.<sup>38</sup>

In Fitzgerald et al.,<sup>37</sup> three collagen-based 3D scaffolds were developed to evaluate a more physiologically relevant model of *in vitro* prostate cancer-bone metastasis. Two of the models incorporated different concentrations of nanohydroxyapatite (nHA) to recapitulate the the inorganic component of bone in order to improve their osteoconductive and osteoinductive capacity.<sup>37</sup> The third collagen scaffold incorporated glycosaminoglycans, which are involved in cell adhesion, migration, proliferation, and differentiation.<sup>37</sup> The 3D collagen models were compared to their 2D counterparts for their ability to support



transfection of gene-based delivery vehicles for RNAi therapies in prostate cancer. The 3D models behaved more like *in vivo* tumors as shown in their gene expression, resistance to docetaxel treatment, and cell proliferation than 2D cell cultures.<sup>37</sup> To further improve the efficacy of the collagen models for prostate cancer, in a separate study Fitzgerald et al.<sup>39</sup> then co-cultured osteoblasts with prostate cancer cells in collagen-nHA scaffolds. The co-culture allowed direct cell-to-cell contact between osteoblasts and prostate cells as the interaction occurs *in vivo*, therefore producing a more clinically relevant model. Cell proliferation was reduced as compared to 2D cell culture, and MMP9, a marker of prostate cancer invasiveness, was enhanced. The investigators conclude that their ability to co-culture cancer and bone cells in a more native *in vivo*-like arrangement will accelerate development of novel next-generation cancer therapeutics.<sup>39</sup>

Many collagen/breast cancer models have also been developed. For example, Sameni et al. used a 3D co-culture called MAME (mammary architecture and microenvironment engineering) to investigate the progression of pre-invasive breast lesions into an invasive phenotype.<sup>40</sup> The 3D model was constructed with a base of multiple layers of collagen I covered with a basement membrane layer. On top of this, cells were seeded and then covered with another layer of basement membrane. This model was unique in the addition of a temporal parameter using live cell imaging to monitor real-time cell-to-cell interactions. The MAME co-culture extended the time available for analysis of tumor microenvironmental changes and tumor growth, invasion, and proteolysis, compared to 2D culture.

Another collagen breast cancer model developed by Chen et al.<sup>41</sup> examined the malignant phenotypes of MCF-7 cells in both 3D and 2D culture. The team investigated pro-angiogenic growth factor secretion, matrix metalloproteinase (MMP) transcription, epithelial to mesenchymal (EMT) transition phenotype, and cancer stem cell property acquisition. The investigators found that cells cultured in the 3D collagen scaffolds had significantly increased malignant phenotypes versus 2D culture. MCF-7 cells in 3D culture overexpressed pro-angiogenic factors and MMPs, which are associated with cancer ECM degradation, metastasis and angiogenesis. Another important factor the 3D culture provided was the hypoxic conditions as found *in vivo*, which aids in metastatic transformation and tumor invasion. Overall, collagen scaffolds enhanced tumorigenicity of the cells to better mimic *in vivo* conditions, and gave insight into metastatic processes.

In Hambach et al., collagen I scaffolds successfully facilitated growth of tumors with complex morphology akin to that found *in vivo*.<sup>42</sup> Single human tumors of different cancer types (breast, kidney, and skin) were generated using human tumor cell lines on collagen I scaffolds.<sup>42</sup> The models underwent multiparametric analysis after exposure to cellular immunotherapy. The resulting 3D tumor models resembled clinical tumors in their morphological aspects, growth patterns, and heterogeneity of target antigen expression and growth niche composition.<sup>42</sup> The corresponding 2D cultures lacked the similarity to clinical models and differed in gene expression and intracellular signaling from the 3D tumor models.<sup>42</sup> The ability to survey several parameters in treatment responses provided a valuable test of the efficacy of immunotherapeutics. Overall, 3D collagen models have thus proven very useful for cancer modeling.

**Chitosan, alginate, and chitosan-alginate (CA) blends**—Chitosan and alginate are natural polymers that have a structure similar to glycosaminoglycans, a principal constituent of ECM.<sup>43</sup> Alginate polymers can be crosslinked to form hydrophilic hydrogels that support high cell loading densities and viable long-term cultures.<sup>44</sup> Several cell types have been cultured in alginate scaffolds such as neuronal cells, osteoblasts, chondrocytes, and myoblasts.<sup>44</sup> Chitosan is a natural polymer that is renewable, osteoconductive,<sup>45</sup> biodegradable, biocompatible, non-antigenic, nontoxic, and biofunctional.<sup>46</sup> However, chitosan, like alginate, lacks the proper mechanical strength for hard tissue engineering applications, such as bone modeling. Therefore, these materials are commonly reinforced with various ceramic phases like ollastonite, hydroxyapatite, and beta tricalcium phosphate. Chitosan can also be blended with natural polymers like silk, gelatin, and alginate.<sup>45,46</sup> The combination of chitosan and alginate produces a mechanically strong chitosan-alginate (CA) scaffold with potentially osteoconductive properties.<sup>45</sup> CA scaffolds have been demonstrated to replicate *in vivo* tumor microenvironments for human glioblastoma and hepatocellular carcinoma more effectively than 2D or Matrigel cultures.<sup>43</sup> Blended CA scaffolds are easily dissolved in cell compatible solutions for cellular release and analysis and have long-term stability in cell culture media.<sup>43</sup>

In one study, prostate cancer cells were cultured on CA 3D scaffolds, 3D Matrigel matrix, or in 2D tissue culture dishes for 15 days and evaluated by proliferation assays and scanning electron microscopy (SEM) for morphology assessment.<sup>43</sup> Prostate cancer cells, with or without primary lymphocytes, in Matrigel formed spheroids, similar to what is seen *in vivo*, while cells on the 2D surface remained flat, linear and elongated. The investigators also performed longitudinal studies on the 3D models to evaluate stability and cell growth over a long time (55 days) using live-cell fluorescence imaging. Matrigel degraded at 7 days and could not be further imaged, even with different seeding densities. However, robust CA scaffolds remained intact and supported large tumor spheroids after 55 days. The investigators summarize that the CA scaffolds generated a more clinically relevant response than the 2D cell cultures and that CA 3D scaffolds are an improved prostate cancer *in vitro* tumor model than 3D Matrigel matrix or 2D culture. Wang et al.<sup>47</sup> also used 3D CA scaffolds with prostate cancer cells and confirmed the utility of these for long-term culture and 3D spheroid formation that mimicked *in vivo* morphologies. Gene expression of ECM and EMT gene markers was upregulated in the 3D CA scaffold. The CA scaffolds were also beneficial models for predicting *in vivo* targeting efficiency, which was not true in 2D.<sup>47</sup> In sum, the CA 3D scaffolds are able to sustain long-term culture of prostate cancer cells and support immune cell interactions, suggesting that this type of model could be applied to screening immunotherapies in an array of cancers.

**Hyaluronic acid**—Hyaluronic Acid (HA) is a structural molecule that is ubiquitously distributed in the ECM and provides tissues with many of its biomechanical properties.<sup>24</sup> It does not invoke an immune response, is non-toxic, and is readily synthesized and metabolized via normal physiological pathways.<sup>24</sup> HA can be cross-linked to form hydrogels with different mechanical properties, depending on the desired application.<sup>24</sup> For cells to adhere and migrate, the substrate they grow on must have a certain stiffness and cell-matrix adhesion proteins. For HA hydrogels the compressive modulus can range from



9.3kPa-22.6kPa.<sup>28</sup> Narayanan et al.<sup>22</sup> tested matrix composition and stiffness of 3D, HA-based hydrogels for applications in BM MSCs and myeloma cell cultures. The percent survival of cells grown on medium stiffness hydrogels was higher than lower or higher stiffness samples, but cell viability was the same across all stiffness levels. The tunability of the HA 3D scaffolds is advantageous since they can be tailored to one's desired tumor model. T. Pan et al<sup>48</sup> investigated renal cell carcinoma (RCC) bone metastasis and found that cells remained viable over 24 days and formed spheroids in HA hydrogels, while in 2D culture the cells began to die after 2 days.<sup>48</sup> Also, some genes strongly associated with RCC metastasis mechanisms and bone homeostasis were significantly higher in 3D versus 2D cell culture, again highlighting the utility of 3D culture.

Xu et al.<sup>49</sup> created a novel HA hydrogel by designing a double network poly(N<sub>e</sub>-acryloyl L-lysine)/hyaluronic acid (pLysAAM/HA) hydrogel to better replicate the breast tumor microenvironment found *in vivo*. The addition of the polymer improved the structural integrity and mechanical properties of the HA hydrogel allowing for long term. The elastic moduli of three pLysAAM/HA hydrogel formulations were  $11.4 \pm 0.8$ ,  $31.5 \pm 1.5$  and  $46.3 \pm 3.2$  kPa, which are comparable to normal breast tissue, which can vary from 17.5 to 271.8 kPa. The pLysAAM/HA hydrogels also had good biocompatibility, and supported breast cancer cell growth and proliferation. When compared to their 2D counterparts, the hydrogels enhanced tumorigenic activity that mimicked *in vivo* behavior. The versatility of HA hydrogels with the ability to incorporate different materials and polymers makes them desirable for tumor engineering.

**Bacterial nanocellulose**—Bacterial nanocellulose (BNC) is a natural, nanofibrous polymer that is synthesized by the bacterium *Acetobacter xylinum* in virtually any microarchitecture and porosity during the bacteria fermentation process.<sup>50</sup> BNC 3D cultures have unique and desirable properties including biocompatibility, high water affinity, unique *in vivo* integration due to non-degradability, and long shelf-life stability (over a year).<sup>51</sup> BNC is inexpensive and has been used for 3D culture for cell types including neuroblastoma cells, MSCs, and osteosarcoma cells.<sup>51</sup> It has also been used successfully in *in vitro* tumor engineered models with PC3 prostate cancer PC3 cells, renal cancer cells, and breast cancer cells. Xiong et al.<sup>50</sup> used micropatterning to create macropores to overcome cell penetration issues in BNC. The patterned microporous BNC scaffolds promoted human MDA-MB-231 breast cancer cell proliferation into the scaffold, cell adhesion, and spreading. C. Gorgun et al.<sup>52</sup> investigated the role of hypoxia on viability, morphology, and stemness of osteosarcoma stem cells using 3D bacterial cellulose scaffolds. The 3D bacterial cellulose scaffolds were able to support tissue microarchitecture, thus mimicking tumor structures and replicating *in vivo* conditions. The investigators discovered that hypoxia had no negative effect on cancer stem cell viability and that the subpopulation of osteosarcoma cells seen preserved their stemness and pluripotency. In sum, BNC 3D scaffolds are a new biomaterial currently being explored because of their potential in tumor and tissue engineering for long-term studies, high-throughput drug screening, and understanding cancer cell behavior.

**Matrigel**—Matrigel is a hydrogel that is derived from mouse tumor basement membrane, which is mostly constructed of collagen and laminin.<sup>53</sup> Matrigel has been used in 2D culture

as a thin layer, and in 3D culture as a hydrogel. It is typically mixed with other materials like collagen for 3D cell culture applications, since it has poor mechanical properties that may not be suitable to recapitulate the tumor microenvironment long-term by itself.<sup>54</sup> For example, in studies by Kurup et al.,<sup>55</sup> two methods of culturing breast cancer cells are explored: the overlay protocol (OP) and the embedded protocol (EP). For the OP, cells are cultured on a thin film of ECM made of Matrigel and collagen I. In the EP, the cells are completely submerged in this matrix. The authors found that even with identical ECM composition, the cells grown using the two different protocols were phenotypically different and differed in gene expression. Therefore, this work again demonstrated that a 3D physical environment and its mechanical properties are important factors in tumor formation and support. Eberle et al.<sup>56</sup> used Matrigel for the investigation of cancer cell invasion in the formation of metastasis as it pertains to cancer treatments using X-irradiation therapy. The use of X-irradiation has been postulated to stimulate cancer cell motility/invasion and metastases by activating defined cellular and molecular mechanisms in the irradiated tissue microenvironment. The investigators used a commercially available 3D Matrigel model, an 8- $\mu$ m pore size BD BioCoat™ Matrigel™ Invasion Chamber with human adenocarcinoma and prostate cancer cell lines. Each experiment in 3D also had a 2D counterpart done in parallel for comparison. Cell motility experiments using the Matrigel coated membranes assessed cell migration changes in response to irradiation. The investigators found that three of the four cancer cell lines grown in 3D, unlike the cancer cells grown in 2D, had a higher motility rate that better replicated *in vivo* conditions. However, the investigators found no significant effects of X-irradiation in either 2D or 3D cell cultures. Because previous studies using different cancer cell lines indicated that X-irradiation can affect migratory potentials<sup>57</sup>, it appears that cancer cell migration response to X-irradiation may be dependent on tumor type, and dose and type of X-irradiation. The ability to model different tumor types using 3D models is critical in discovering effective treatments for different types of cancers, and there is great benefit in using Matrigel in these models.

**Bone-Cell Derived ECM and Native Bone**—A recent area of interest in 3D cell culture is the use of native bone either with or without other biomaterials. To model the interactions between myeloma and the BM ECM, de la Puente et al.<sup>58</sup> created 3DTEBM: 3D tissue engineered BM derived from BM supernatants from myeloma patient samples. These cultures used myeloma cells, stromal cells and endothelial cells. This model allowed for realistic oxygen and drug gradients, supported proliferation of tumor cells, and induced more drug resistance than 2D or commercial 3D tissue culture systems. Similarly, Templeton et al. used tissue fragments extracted from femoral bone via a rongeur without further processing, co-cultured with breast cancer cells to investigate cancer cell migration, colonization, and proliferation.<sup>59</sup> The investigators discovered that the use of the bone fragments supported relevant cell types in the human bone microenvironment and were able to sustain cell viability. However, the 3D cultures had a short culture time (48–72 hours) which may reduce their applicability.

Another method of using native bone for 3D culture is decellularized bone matrix. Decellularization removes cells from the tissue of interest while preserving the structural and functional proteins of the ECM framework.<sup>60</sup> The decellularized tissue can then be seeded

with the cells of choice. Reichert et al.<sup>61</sup> used decellularized mineralized matrix from human osteoblasts to create 3D prostate cancer models. The 3D model contained normal bone ECM proteins like osteocalcin, osteonectin, and osteopontin, all of which prostate cancer cells would encounter during bone metastasis. The use of native tissues and biomaterials is a novel avenue of research, but challenges to overcome include reproducibility, long-term culture and access to raw patient materials.

### Synthetic Biomaterials

In addition to natural biomaterials, there are also synthetic biomaterials. Similar to natural biomaterials, synthetic biomaterials can be synthesized with specific mechanical and diffusion properties, and functionalized with ligands to promote cell-cell and cell-matrix communication and adhesion.<sup>62</sup> Synthetic polymers have advantages over some natural materials because their properties are more easily controlled and they can be modulated to display specific responses to external stimuli like pH, temperature, salinity, or concentrations of small biomolecules.<sup>62</sup> Here we describe some of the commonly used synthetic biomaterials.

**Poly(ethylene) glycol: PEG**—PEG is an inert, synthetic polymer popular for use in 3D cell culture because of its biocompatibility, high water content, tissue-like elasticity, and easily tunable biophysical and biomechanical properties.<sup>54</sup> The mechanical stiffness of PEG hydrogels can be manipulated by polymer network cross-linking density for different applications and integration into different tissues. PEG can also integrate different cell-responsive motifs like integrin ligand RGD peptides, to promote cell attachment, or MMP-sensitive peptides, to allow cell-mediated matrix remodeling.<sup>54</sup> Taubenberger et al.<sup>63</sup> used MMP-degradable biohybrid PEG-heparin hydrogels to investigate the effect of biochemical cues on the microenvironment of early cancer events. Their team used this PEG-based hydrogel successfully for the design of tumor angiogenesis microenvironments of breast and prostate cancer cells with vascular endothelial cells and MSCs. PEG hydrogels were found to be useful in examining morphological changes, invasion, proliferation, and tumor angiogenesis *in vitro*. PEG can also be 3D printed for precise control of matrix/scaffold architecture for improved scalability and reproducibility. Zhu et al. designed 3D bone matrices by computer-aided design (CAD) software and printed them with a tabletop stereolithography-based 3D bioprinter.<sup>64</sup> PEG and nHA hydrogels were fabricated at different weight percentages to change matrix stiffness and printed in three 400  $\mu\text{m}$  layers. The 3D-printed PEG-multicellular based hydrogels supported spheroids that resemble natural tumor structure found *in vivo* while 2D cultures formed monolayers.

**POLY( $\epsilon$ -CAPROLACTONE): PCL**—PCL is a commonly used polymer for tissue engineering due to its biocompatibility, mechanical and structural properties, ease of fabrication, and long-term biodegradability.<sup>65</sup> PCL can be fabricated to have the architecture and stiffness similar to a human tumor, and encourages cells to deposit their own matrix, thereby facilitating cell attachment and growth in 3D.<sup>66</sup> Swaminathan et al.<sup>67</sup> found that tumor stiffness correlates with invasiveness of cancer cells. The investigators observed that cancer cell lines displayed varying degrees of stiffness throughout a given population and this variation directly correlated to metastatic progression.<sup>67</sup> Tumor cell stiffness values are

highly dependent on the tissue they are growing in. Tissues in the body have an elastic modulus that ranges from approximately 50 Pa in neural tissue to 4 GPa in bone tissue. The ease of tunability of their physical properties, such as stiffness, and ease of integration with other biomaterials and polymers, makes PCL a desirable material for scaffolds. Sieh et al.<sup>68</sup> designed a model using two constructs to study prostate cancer bone metastasis: a soft PEG hydrogel disc seeded with LNCaP prostate cancer cells adjacent to a hard tissue-engineered bone (TEB) construct made from mPCL-TCP (medical grade polycaprolactone –tricalcium phosphate) that was seeded with primary human osteoblasts which were grown in osteogenic media and produced mineralized bone matrix. The PEG hydrogel allowed for the prostate cancer cells to form multicellular masses that resembled avascular tumors, and the TEB produced osteoblast-derived signals to the cancer cells. Similar to the Kwon<sup>36</sup> study on silk, this study also demonstrated osteomimicry by prostate cancer cells. The combined PEG and TEB co-culture model, unlike traditional 2D cell culture models, supported the regulation of androgen-responsive genes and increased the expression of genes associated with bone growth factors, matrix proteins, and bone remodeling enzymes, which are all also relevant to prostate cancer bone metastasis. This biphasic model has the potential to provide a bone microenvironment platform that can advance translational studies and high throughput testing of cancer therapies.

Balachander et al. also investigated the use of PCL 3D scaffolds as a model for breast cancer metastasis.<sup>66</sup> MDA-MB-231 cells were grown on PCL scaffolds and compared to *in vivo* tumorigenicity and metastatic potential using gene expression microarrays, structural and mechanical characterization, cellular morphology using confocal microscopy, and cellular migration and invasion assays. PCL scaffolds resembled the topographical and mechanical properties of breast tumors due to their low elastic modulus and open pore architecture as compared with traditional 2D cell culture. Expression of gene genes related to metastatic initiation, progression, and colonization were similar for cells cultured on PCL scaffolds and cells *in vivo*, but this was not observed for 2D culture samples. The 3D cultured cells also had a strong pro-inflammatory gene expression signature, which could become a potential target for therapy since inflammation has been associated with cancer.<sup>66</sup> PCL scaffolds increased the metastatic properties of breast cancer cells, therefore providing a better platform for the screening of therapeutics than traditional 2D cell culture.<sup>66</sup> Thus, PCL scaffolds hold promise in serving as a platform for designing effective treatments and aiding in the understanding of cancer progression.

Similarly, in Holzapfel et al., PCL scaffolds coated with calcium phosphate promoted cell adhesion and osteogenic differentiation *in vitro*, and bone formation *in vivo*, to investigate advanced prostate cancer bone metastasis by creating a pre-metastatic bone marrow niche.<sup>69</sup> In this study, PC3 prostate cancer cells were grown in static 3D culture on PCL scaffolds for 4 weeks and then transferred into a bi-axial bioreactor for another 4 weeks to increase fluid dynamics. After 8-weeks, the samples were grafted into mice. The researchers were able to increase metastatic capacity of the PC3 cells injected into the left cardiac ventricle of mice. This was accomplished by providing a humanized niche using PCL scaffolds seeded and cultured with human mesenchymal progenitor cells, for the PC3 cells to home to and develop macro-metastases in. In another study, Fong et al. grew human Ewing's sarcoma TC-17 cells on 3D PCL scaffolds *in vitro* and found that this cultivated a well-differentiated

Ewing's sarcoma-like phenotype similar to that found *in vivo*.<sup>70</sup> The model was compared to its 2D culture counterpart currently used as a pre-clinical gold standard for assessing drug efficacy. In the 3D PCL model, biochemical and morphological features, growth kinetics, and protein expression mirrored what is seen *in vivo*. On the other hand, 2D cell culture models poorly replicated Ewing's sarcoma tumor biology.<sup>70</sup> In two clinical trials the investigators conducted, the response to the antagonist used as a therapeutic had an unremarkable response in 2D cell culture but demonstrated tumor regression in a subset of EWS patients in the clinical trial. The 3D model appeared to be better at reproducing an *in vivo*-like response to therapeutics thus could potentially be better at identifying therapeutics for greater patient efficacy.

#### **Poly(amino acid)-based polymers and poly(lactide-co-glycolide):(PLG)—**

Poly(amino acid)-based polymers are a new class of biomaterial used in tissue engineering because of their structural diversity, and because they are easy to synthesize and have chemical, biological, and mechanical properties that can be easily manipulated.<sup>62</sup> Zrínyi et al. used poly(aspartic acid)-based (PASP) hydrogels as 3D scaffolds to investigate osteoblast adhesion and proliferation in osteosarcoma.<sup>62</sup> PASP is a new scaffold material that has been shown to accelerate tissue regeneration and replace natural ECM by filling in damaged areas.<sup>62</sup> Lynch et al.<sup>71</sup> used PLG 3D models with and without hydroxyapatite to investigate osteogenic differentiation and OPN expression of BM-MSCs under controlled mechanical stimulation and breast cancer cell-derived paracrine factor influences. PLG scaffolds were cyclically mechanically compressed, which resulted in increased OPN expression in BM-MSCs undergoing osteogenic differentiation as compared to the static 2D cultures. Furthermore, when the samples were exposed to tumor-derived soluble factors, OPN expression was further enhanced. The enhancement indicates that OPN might not only be a factor in bone cell adhesion to the ECM, but also a primer for breast cancer cell adhesion or may in other ways support tumor progression.

#### **Scaffold-Free, Bioreactors, and Dynamic Cultures**

As described above, 3D culture systems are widely produced using natural and synthetic biomaterials. However, another emerging option is scaffold-free 3D culture, where cells grow typically in a sphere without an externally-applied biomaterial for support. The cells grown in spheroids are in different proliferative and metabolic states similar to *in vivo* conditions which improves testing and designing of drug therapies. These organoid cultures typically do not grow as large as cultures in scaffolds, and they risk becoming hypoxic in the center if they grow too large. Tumor spheroids, often formed using poly-HEMA (non-tissue culture-treated plastic) plates, often start from a single cell suspension and can be generated in a range of sizes depending on application and method.<sup>72</sup> Larger spheroids starting from around 500  $\mu\text{m}$  in diameter often display the cellular heterogeneity found in *in vivo* tumors.<sup>72</sup> Organoid cellular clusters can be derived from primary tissue, embryonic stem cells, or induced pluripotent stem cells. Some are capable of self-renewal and self-organization and exhibit similar organ functionality to the tissue of origin.<sup>73</sup> Organoids can be used for disease modeling, gene editing, personalized therapies, and analyzing stem cell behavior.<sup>73</sup> For example, some 3D breast cancer organoid cultures have been found to be more innately resistant to treatments (neratinib and docetaxel) than 2D cultures.<sup>74</sup> This may be due to

differences in drug diffusion or signaling pathways that govern breast cancer response to drugs. Considerable research is now aimed at designing new assays for 3D tumor spheroid models that better account for the variability in the data and methods, but nutrient diffusion constraints will always limit the size that organoids will be able to reach.

Mechanical signals also play an important role in modulating tumor behavior in the bone microenvironment.<sup>71</sup> Osteoblasts and osteocytes are mechanoreponsive and involved in the regulation of osteoclast differentiation and activity.<sup>71</sup> Consequently, the mechanical loading of the skeleton plays an important role in bone metastasis. Bioreactors are devices developed to mimic *in vivo* physiology. They allow for precise and reproducible control over cellular environmental conditions, such as temperature, pH, flow rate, oxygen nutrient supply, and waste metabolite removal, and can provide mechanical loading to cells.<sup>75</sup> In the vascular compartment the *in vivo* tissue conditions are not static thereby creating a need for a 3D models in bioreactors to better recapitulate tumor progression. Bioreactors for use in bone tissue models apply fluid shear stress thorough porous scaffolds that activate cellular processes like differentiation and proliferation and aid in the perfusion of nutrients and oxygen transport.<sup>76</sup> Another critical factor in bone formation and repair is oxygen concentration. For example, high dissolved oxygen concentrations of 5–10% are needed for mineralization.<sup>76</sup> Bioreactors can supply the mechanical forces of shear stress, pressure, gravity and oxygen concentrations needed to replicate physiological conditions for 3D models of bone. Bioreactors also overcome mass transfer limitations that are associated with static 3D culture.<sup>77</sup> Moreover, perfusion and fluid shear stress affect the developmental and differentiation of stem cells.<sup>78</sup> Ferrarini et al. forced cells to grow in 3D using a dynamic culture bioreactor, called the Rotary Cell Culture System, where paclitaxel-releasing MSCs were found to inhibit the growth of multiple myeloma cells.<sup>79,80</sup> Using novel bioreactors utilizing magnetic levitation and bioprinting method, another group forced cell spheroid formation using co-cultures of fibroblasts and tumor cells.<sup>81</sup> The fibroblasts not only acted as a signaling stromal cell for the cancer cells, but they also produced ECM (collagen and fibronectin) such that the tumor cells were growing in an ECM ball. Although not yet used for cancer in bone, plates known as “3D Biomatrix hanging drop plates” have been used by other oncology researchers to investigate amidino-substituted benzimidazole and benzimidazo[1,2-*a*] quinoline derivatives’ effects on prostate and breast cancer cells in 2D and 3D culture systems.<sup>82</sup> Changes in mechanical forces that can be controlled in a bioreactor potentially can signal pathways and cellular processes critical in understanding tumor formation and metastases. Further studies testing the effects of dynamic mechanical forces on bone metastatic pathogenesis using scaffolds may be useful in understanding how tumor cells respond to mechanical forces.

Microfluidic 3D cell culture systems can also be used to add a dynamic aspect to cultures. Microfluidic 3D cell cultures provide the user with precise control over experimental conditions and enable parallelization and automation, with low variability.<sup>83</sup> These systems can mimic *in vivo* conditions by providing conditions to study cancer cell flow, attachment, transmigration, and colonization with fluid flow.<sup>84</sup> Angiogenesis is critical for tumor growth and metastatic spread to bone and other organs; it is often modeled using microfluidic devices, such as described by Fu et al. to investigate leukemic cell-induced angiogenesis *in vitro*. The team engineered a device with microfluidic channels across a collagen matrix that



would flow gradients of angiogenic factors secreted by leukemic cells that mimicked angiogenic induction.<sup>85</sup> Leukemic cells were cultured with and without BM-MSCs in the 3D microfluidic angiogenesis chip in order to characterize angiogenic sprouting and morphogenesis differences induced by leukemic cells in the BM environment. This model could be useful for the evaluation of drug treatments of individual leukemia patients.

## 2D Versus 3D Culture Comparisons

It has become increasingly accepted that 3D cell cultures are more valuable than 2D cultures due to their capacity to mimic multidimensional tumor structures that are essential for correct cell-cell communication (e.g., integrin or notch signaling) and cell-matrix interaction (e.g., cytoskeletal rearrangements for pulling or pushing). The physical forms of cell cultures influence tumor growth rate, sustained tumor size, drug resistance, dormancy, and other characteristics. Moreover, cells grown in 2D cultures are forced to adopt a sheet-like morphology in which only a fraction of their cell membrane receives signals. By contrast, in a 3D environment, 100% of the membrane receives soluble signals. In 2D culture, cell polarization and phenotypes are abnormal and cell signaling via adhesion kinases, integrins, selectins, or selectin ligands is reduced compared to 3D cultures. This signaling and physical pressure on cells alters cell growth, migration, and apoptosis for both malignant and healthy cells. Still, 2D cultures are advantageous in that they are generally easier to maintain, simple, fast, and less expensive compared with 3D models and cost-intensive animal testing. This is due to the fact that all cells cultured in a 3D environment react differently and exhibit different phenotypic and genotypic properties compared to cells cultured in monolayers.<sup>86</sup> For historical reasons, 2D culture is currently considered the “gold standard” of much biomedical research. Nonetheless, for bone metastatic cancers, as with many cancers, 3D culture methods are increasingly becoming the workhorse in data collection and are necessary to propel clinical and medical research forward.

Among tissue engineering researchers, there is growing consensus that 3D models better recapitulate *in vivo* conditions and cell responses than 2D models do.<sup>53</sup> This review has provided an overview of the basis for this preference. Although academic laboratories have already begun to appreciate the suitability of 3D culture, and many industry and pharmaceutical laboratories are also benefiting from incorporating 3D culture into lab practice for drug development and screening research. This will likely improve the failure rate for oncology compounds entering clinical trials (currently about 90%), which is partially attributable to 2D and monoculture pre-clinical compound identification tests that produced misleading or non-predictive data for *in vivo* responses.<sup>87</sup> Indeed, 3D models have recently shown significant growth in utilization in the pharmaceutical industry as 3D models have become better characterized.<sup>88</sup>

## Conclusions

Animal models have some advantages over 3D systems since they can be used to study cancer spread and metastasis, shear flows/fluid forces, angiogenesis, cancer immunology, and metabolic or systemic influences on cancer all within the same animal. However, 3D models are becoming ever more sophisticated and bioreactors are being incorporated to

better model *in vivo* fluid dynamics and mechanical stress. Moreover, the addition of endothelial cells, adipocytes, and immune cells to 3D cell cultures is now common, and media can be modified easily to study metabolites, hormones, or different energy sources. The understanding of immune responses has become increasingly important in our understanding of cancer and efficacy of drug therapies. The use of 3D cell culture models furthers this understanding by better replicating *in vivo* immune responses. Properties of 3D models used in investigating immune responses can be manipulated by the addition of immune cells and immune cell conditioned media. Modifications of biomaterial surface properties such as hydrophilicity, chemical modifications, roughness, and topography also can be used to optimize 3D models.<sup>89</sup> Compared to *in vivo* models, 3D models are advantageous because inputs such as cell types, cell numbers, soluble factors, and matrices can be controlled more reliably.<sup>36</sup> Moreover, animal models can have limited translatability to human physiology due to differences in mouse and human biology and protein/gene homology. Additionally, adequate animal models that can recapitulate the biology of disease in humans are often lacking. Conversely, 3D models can be completed with 100% human-derived components (scaffold material, media proteins and cells) increasing translational relevancy of the findings and side-stepping issues related to animal ethics (e.g., animal suffering). Overall, 3D tissue engineered models provide researchers with a compromise between 2D and *in vivo* models of bone cancer, creating a bridge for the gap between 2D and *in vivo* research findings.

In terms of cancer in the bone, it is clear that the choices of model system, co-culture cell types, and biomaterials are dependent on the scientific question at hand. For questions where osteolysis is being investigated, osteoclasts are a necessary cellular component. Where inhibition of bone formation is studied, osteoblasts or pre-osteoblasts must be included. Appropriate mechanical stiffness is crucial in osteogenic analyses, as biomaterial stiffness and strength directly relate to osteogenic differentiation.<sup>90</sup> Weighing the advantages and disadvantages of each model system and biomaterial, it is also important to consider the experimental parameters for the system of interest, such as the source of tumor cells, metastatic pathways, and clinical applicability. Considering these parameters is crucial for designing the appropriate *in vitro* model. Typically, it is best to match as many properties as possible to *in vivo* conditions. However, models can be simplified pragmatically and less important cell types can be omitted to increase the usability, efficiency, and value of the model and decrease costs and complexity. There are many variables and options in designing a 3D model; for more information on previously used models and materials, please refer to Table 1.

In summary, 3D models are a promising approach for researching mechanisms and interactions of cancer within the BM microenvironment. 3D models better mimic *in vivo* conditions not only for cancer cells, but also for surrounding stromal cells. Co-cultured 3D models improve the bench-to-bedside translation of research. The use of 3D, co-culture assays, even as secondary screens after 2D assays, will accelerate discovery and decrease wasted time, effort, and materials in the drug discovery arena. New 3D models are currently being developed to reveal novel targets using co-culture/tri-culture systems with cells previously not explored deeply in relation to bone cancer, such as BM adipocytes, immune cells, and osteocytes. The 3D models described here, and in development in our lab and

others, enable more in-depth and accurate investigations into bone cancer. The goal is that these 3D models will assist us in revealing novel mechanisms, detecting biomarkers, designing diagnostics, and developing therapeutics, all with the goal of improving patient prognosis and care, quality of life, and life expectancy.

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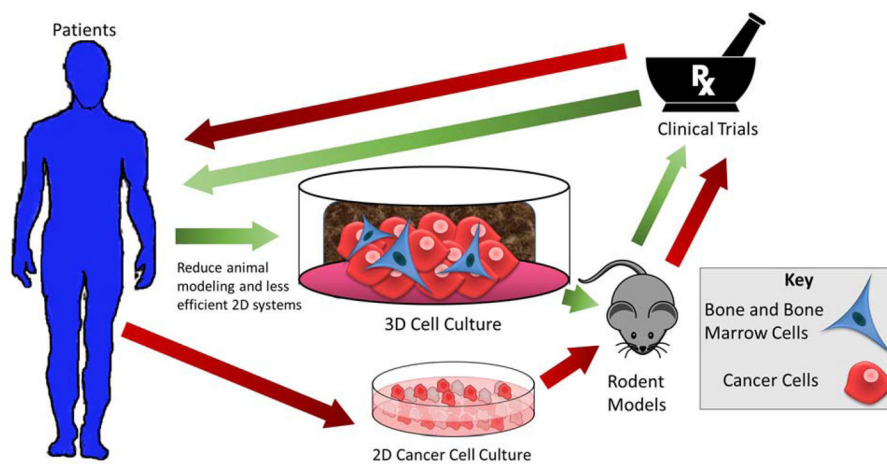


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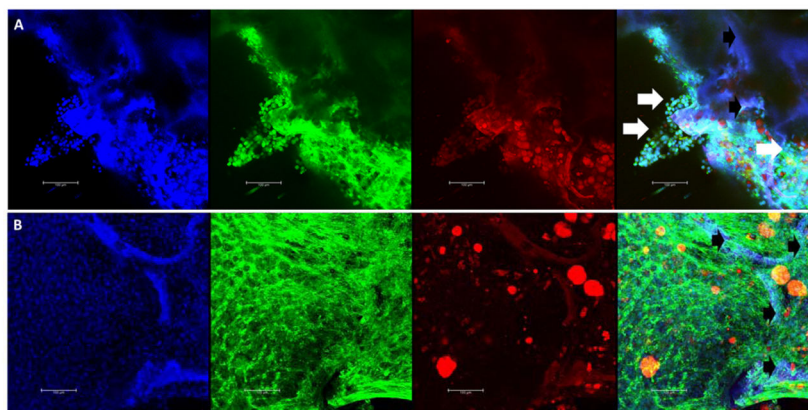


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**FIGURE 1.** Traditional methods of designing and testing therapeutics utilize 2D cell culture, then animal models, and finally clinical trials in patients (longer red pathway). Using 3D cell culture (shorter green pathway) allows scientists to bypass lower efficacy 2D models, minimize large-scale and expensive animal models, decrease false positives, and improve clinical research by identifying promising candidates or therapeutics more rapidly.



**FIGURE 2.**

Confocal Imaging of Silk Scaffold Tissue Engineered Bone-Cancer Model. A heterogeneous population of mouse bone marrow stromal cells, containing mouse pre-bone marrow adipocytes, was seeded onto porous silk sponges, cultured for 9 days in growth media, and then switched to an adipogenic media for 10 days. Next, scaffolds were seeded with human green fluorescent-protein expression (GFP<sup>+</sup>) MM1S myeloma cells (A) or cultured alone (B) for 7 days. Scaffolds were formalin-fixed and stained with DAPI (for nuclei, blue), phalloidin (for actin, green), and Oil Red O (for lipids, red). Cellular phenotype was used to distinguish between green MM1S cells (round with large nuclei) and stromal cells (elongated). Confocal images show a maximum projection of blue, green, red and overlay channels (left to right). Silk is autofluorescent in every channel and thus appears purple in the overlay. White arrows indicate tumor cells. Black arrows indicate silk scaffold. The scale bar represents 100  $\mu$ m in each image. This 3D model of BMAT and myeloma is one example of a 3D model that can be used to investigate the interactions and effects of myeloma on BMAT, and vice versa, in a realistic, long-term co-culture model. Such cultures provide unique insight into interactions between cancer cells and other cells of the bone and bone marrow that can then be applied to drug discovery.

**Table 1**

## Biomaterials used in Tissue Engineered 3D Bone Models

Biomaterial	Scaffold Type	Cancer	References
<i>Natural Materials</i>			
Silk fibroin	Scaffold, hydrogel, mat	MM, prostate, breast, osteosarcoma	12,15,34,35
Collagen	Hydrogel, scaffold	Breast, prostate, osteosarcoma	38,40,91
Chitosan-Alginate	Scaffold	Prostate, glioblastoma, hepatocellular carcinoma	43,45
Hyaluronic Acid	Scaffold, hydrogel	Renal cell carcinoma, MM	22,48
Bacterial nanocellulose (BNC)	Scaffold, hydrogel	Neuroblastoma, osteosarcoma, prostate, renal cancer, breast	50,51
Native ECM	Scaffold	MM, breast	58,59
ECM/cartilaginous matrix/Matrigel	Scaffold, hydrogel	Breast	55
Chitosan (with or without HA or collagen)	Scaffold	Breast	92
Cell sheets over medical-grade polycaprolactone-tricalcium phosphate	Scaffold	Prostate	93
<i>Synthetic Materials</i>			
Poly(ethylene glycol) (PEG)	Hydrogel	Breast, prostate	64,68,94
Poly( $\epsilon$ -caprolactone) PCL	Hydrogel	Breast, prostate, osteosarcoma, Ewing's Sarcoma	66,69,70
Poly(amino acid)-based polymers	Hydrogel	Osteosarcoma	62
PLG non-mineralized PLG mineralized with HA	Scaffold	Breast	95

*Abbreviations:* BM, bone marrow; ECM, extracellular matrix; HA, hydroxyapatite; MM, multiple myeloma