Osteosarcoma (OS) is the most common primary malignant tumor of bone that mainly affects children and adolescents. The currently available therapies are not effective and the search for new OS anticancer drugs is extremely urgent. Understanding the mechanisms that underlie the tumor progression, invasion, and metastasis is an essential step toward effective cancer therapies. Tissue engineering has given a great contribution to the development of reliable and cost-effective platforms for drug screening and validation through 3D in vitro models that more faithfully mimic the in vivo pathophysiology than conventional 2D models. The progress in the field of functional and biomimetic biomaterials in the development of 3D tissue models has provided tools for a close recapitulation of the highly complex and dynamic tumor microenvironment. This review focuses on the most recent advances in 3D in vitro osteosarcoma models, highlighting the crucial role of the extracellular matrix and stromal cells in tumor progression, how they contribute to drug resistance and disease prevalence, and the future pathways toward an effective and personalized model for drug screening and validation.

1. Introduction

Cancer is a complex disease characterized by genetic disorders that are involved in abnormal cell growth and heterogeneous pathophysiology. Osteosarcoma (OS), a rare primary malignant disease, is the most common type of bone cancer that mainly affects children and adolescents, being also found with frequency in adults over 40.[1] Primary OS mainly arise in the long bones of the extremities, with higher incidence in the distal femur, proximal tibia, and proximal humerus.[2] This tumor commonly occurs in the metaphysis, named growth plate of the long bones where the most proliferative cells are located, and preferentially metastasize to the lung both at an initial and later stage.[3,4]

OS origin is attributed to several epigenetic and/or genetic alterations in mesenchymal stem cells (MSCs) or MSC-derived osteogenic lineages, such as mutation of p53 and retinoblastoma tumor suppressor gene inactivation, under the influence of specific bone microenvironment signals. The accumulation of these alterations leads to the emergence of cancer stem cells (CSCs) subpopulations with self-renewal and differentiation capacity, MSC markers expression and increased DNA repair ability, sustaining tumor growth, recurrence, metastasis and drug resistance.[5]

The available therapies for OS include neoadjuvant chemotherapy with standard cytotoxic drugs such as doxorubicin, methotrexate, and cisplatin, followed by surgical resection of the primary tumor and adjuvant chemotherapy.[6] Based on National Cancer Database Report 1985–2003, these therapies are not effective, yielding a 5-year survival rate of 53.9% and besides that, patients with metastatic or recurrent disease have an extremely low survival rate (<30%).[7,8] The most critical issue that underlies these values is chemoresistance, for which the understanding of the molecular mechanisms in cancer may produce better clinical outcomes.[3] Nonetheless, the discovery of new anticancer drugs and effective therapies for OS is an emergency. The low OS prevalence and high tumor heterogeneity have delayed the application of innovative treatments with clinical evidence and limited the investment of the pharmaceutical industry. In this sense, it is crucial to develop new in vitro platforms that truly mimic the pathophysiology of the tumor.

The physicochemical and histopathological properties of the tumor microenvironment are essential for the stimulation of biological functions mediated by cell signaling. In this context, the extracellular matrix (ECM) exhibits a key role depending on its structural features, including the mesh of proteins, stiffness/viscoelasticity, adhesion motifs density, and biophysical properties.[9] It means that although intracellular signaling is autonomous, the extracellular microenvironment is responsible for the regulation of important signaling pathways involved in cell survival through the activation of cell membranes receptors (e.g., integrins).[10] So, the in vitro models of tumor-induced disease developed for biological research need to closely mimic the native microenvironment in order to improve the tumor biology knowledge and the evaluation of drugs efficacy.

The current most used in vitro models, the 2D cell culture, are simple and fast systems, but fail regarding the faithful recapitulation of the complex biological pathways of human tissues due to limited cell–cell interaction and ECM secretion which has negative outcomes at gene and protein expression level of cell surface receptors, growth factors, and structural macromolecules.[11,12] Thus, 3D tissue engineering constructs have been proposed as...
they can balance the realism and the simplicity, artificially mimicking key aspects of any human tissue and its microenvironment and, furthermore, reducing the use of animal models. The microenvironment engineering both for healthy and tumor tissues is being developed using materials that resemble the ECM components. Scaffold-based platforms, like hydrogels and sponges, applying such materials, as well as spheroids deprived of a non-native matrix, have been demonstrated to mimic tissue natural structure. Numerous types of biomaterials have been used to recreate the ECM in 3D in vitro models, from materials of natural origin like the solubilized basement membrane preparation extracted from the Engelbreth–Holm–Swarm (EHS) mouse sarcoma (e.g., Matrigel), collagen, gelatin, and silk fibroin, to synthetic polymer-derived materials such as poly(ethylene glycol) (PEG), polycaprolactone (PCL), poly(lactic acid) (PLA), among others. Although a large variety of biomaterials are already in use for in vitro disease modeling, there is a huge margin of progression in order to optimize or search for new ones, specially taking into account the current focus in personalized medicine.

This review focuses on the most recent advances in 3D in vitro bone cancer models, highlighting the crucial role of the biomaterial composition and biophysical cues in mimicking the bone tumor microenvironment and its effectivenss regarding the recapitulation of drug kinetics. Differences in cell behavior, drug resistance, and disease prevalence will be discussed in terms of strengths and limitations of up-to-date in vitro bone cancer models. In addition, we will underline the next challenges and future pathways that must be addressed toward an effective and personalized 3D in vitro model for osteosarcoma.

2. Role of Microenvironment in Tumor Pathophysiology and Therapeutic Response

Until a few years ago, it was believed that only the autonomous properties of cancer cells were responsible for the development of malignant tumors, as discussed in the six hallmarks of cancer proposed in 2000 by Hanahan and Weinberg. However, several studies performed throughout this century show that the complex and dynamic microenvironment where the cancer cells reside, composed of an ECM, stromal cells, soluble factors, and biophysical features, is actively involved in tumor initiation, progression, invasion, and even in drug resistance. The importance of this crosstalk between tumor cells and its microenvironment components was acknowledged over 100 years ago by Paget et al. in the famous “seed and soil” hypothesis.

Similar to what is verified in a normal wound healing process, the microenvironment in a tumor is composed of a heterogeneous population of reactive stromal cells that promote several events such as neoangiogenesis, ECM remodeling, tumor proliferation, metastasis, and drug resistance mechanisms. In these processes, tumor cells recruit host stromal cells, including fibroblasts, endothelial cells, immune cells (macrophages, dendritic cells, and T cells), MSCs, and others (Figure 1). The dynamic tumor-stromal cell communication, directly or by paracrine signaling, assured by growth factors (e.g., transforming growth factor-β1 [TGF-β1], vascular endothelial growth factor [VEGF], fibroblast growth factor [FGF], platelet-derived growth factor [PDGF]), chemokines (e.g., interleukin [IL]-6, IL-8, stromal cell-derived factor-1), and other soluble factors, plays an essential role in tumor growth, invasion, metastasis, and chemoresistance. In a tumor microenvironment, the excessive cell proliferation results in spatiotemporal depletion of oxygen, generating hypoxia regions that drive a process named “angiogenic switch” through the overexpression of
pro-angiogenic factors.\cite{24} The new vascular network not only supplies the oxygen and nutrients essential for tumor survival but also provides a route for tumor cell extravasation, supporting the tumor development and key events of the metastatic cascade and drug resistance.\cite{25}

Although the importance of heterotypic cell–cell communication is acknowledged, in vivo cellular behavior is highly dependent on its interaction with the ECM, a complex and dynamic network of proteins and polysaccharides that maintains the tissues' integrity, providing them structural support.\cite{9,26,27} The protein portion of the ECM mainly includes collagen, elastin, laminin, and fibronectin, being its spatial distribution dependent on tissue type.\cite{28} For example, in bone matrix, collagenous proteins are the major constituent ($\approx 90\%$), contributing to the tensile stiffness and strength when the tissue is exposed to high strain levels.\cite{29} This protein network integrates negatively charged polysaccharides produced by the cells which fill the interstitial space by the formation of glycosaminoglycans (GAGs) and proteoglycans, contributing to the compressive resistance of the tissues.\cite{27} It is within this hydrophilic microenvironment where essential soluble factors such as nutrients, growth factors, and other signaling molecules keep biologically active, participating in most of the metabolic pathways and controlling cell behavior over time. The dynamic interaction and structural arrangement of the ECM is closely related with the mechanical stiffness of the tissue and, in turn, with its biological function.\cite{9} Native tissues show mechanical properties that vary from effective elastic moduli of 0.1 kPa in "soft" neural tissues to effective elastic moduli of 20 million times higher in stiff ("hard") bony tissues.\cite{30} During tumor development, the tissue homeostasis is destabilized by abnormal ECM remodeling, as a result of the overexpression of protease-like matrix metalloproteases (MMPs).\cite{31} Such enzymatic degradation breakdown the connective tissues, playing a crucial role in tumor development, invasion, and metastasis. For example, high levels of MMP-2 are verified in several cancer types, including OS, and are associated with poor prognosis and enhanced invasiveness.\cite{32}

Understanding the dynamic state of the tumor microenvironment regarding the homotypic or heterotypic cell–cell interaction and spatiotemporal regulation of ECM components has been a huge challenge over years. In this sense, the faithful recapitulation of the in vivo microenvironment has been a focus of the scope of tissue engineering.

3. Breaking the In Vitro Disease Models’ Impasse

The development of disease models has been the focus of biomedical research with the goal of providing a simple, fast, and cost-effective platform for drug development and screening. In the field of cancer research, the pretensions have been the same given the emergency of discovering new drugs and developing new therapies. However, mimicking the tumor environment has been challenging due to the complexity of the ECM and the cellular dynamism.

The understanding of tumor microenvironment was primarily recapitulated in 2D monolayer cell cultures from which most of the current knowledge was obtained. Unfortunately, these in vitro models have some flaws in mimicking the biological characteristics of the microenvironment and signaling cascades.\cite{12} The structural changes to which cells are subjected in 2D models, result in cell–ECM adhesion only on a half of the cell that is in contact with the flat dishes and cell–cell contact is established only in the horizontal plane. This abnormal cellular adhesion has
an important effect in cell function due to unnatural apical-basal cell polarity. This is reflected in the lack of predictivity of these models and in the misleading results obtained in vivo, contributing to the increase of failure rate of new drug discovery in clinical trials.

Over the last few decades, the development of in vivo models, including genetically engineered and cell lines injected or tissue xenotransplanted in mice, has been the main approach to complement the information provided from the monolayer cultures. Although animal models predict biological relevant pharmacokinetic responses, their metabolic and physiological differences from humans hamper the exact recapitulation of the human diseases. Even though in vivo models reduce the drug inefficacy, about 90% of the drugs fail during clinical trials, resulting in an expensive period of clinical development without subsequent effective therapy and also financial return. Furthermore, the high number of animals required during a preclinical study remains an ethical problem, besides being labor-intensive and expensive. Thus, it has become crucial to develop a more predictable in vitro model that can mimic the human in vivo cellular behavior in a more realistic way.

Recently, 3D cell culture models have been a focus for this purpose, once it has demonstrated a more faithful recapitulation of the in vivo microenvironment, modeling the dynamic cell–cell and cell–ECM interactions. In these models, the cells are frequently embedded into a matrix where they can acquire its native polarity and establishes natural interactions with other cells and ECM components, overcoming the main drawbacks of 2D monolayer cultures. In 3D models, the cells can exhibit a specific behavior to the stiffness that is totally different from that verified in 2D cultures, which is reflected into more realistic therapeutic responses. A simple example is the effect of substrate stiffness in cell responsiveness to external adenosine triphosphate (ATP) through calcium signaling pathway, dependent of cell–cell interaction via gap junctions. Also, the spatial gradients and pharmacological kinetics due to constraints to the transport and distribution of soluble factors are accurately mimicked in 3D cultures, in contrast to the free and rapid diffusion in 2D cultures.

The increased precision of 3D cell cultures that may result in a greater predictive value for clinical outcomes has stimulated its application in drug discovery. In fact, 3D in vitro tumor models enable to culture cells in a more physiologically relevant environment than 2D models, providing fundamental insights in cell proliferation, differentiation, gene, and protein expression, response to stimuli, and drug sensitiveness. Besides the structural support, the importance of the ECM in cell response to anticancer therapies has been related with drug availability and changes in drug target expression or intrinsic mechanisms of cellular resistance. The ECM remodeling mechanisms of cancerous tissues to a stiffer network usually contribute to drug resistance. In this sense, some reports have demonstrated that cancer cells cultured into a 3D scaffold are less sensitive to drug than its 2D counterparts, and scaffold stiffness has a role in pharmacokinetics and drug resistance. The crucial role of integrin-mediated transmembrane receptors as chemotherapeutic response modulators is also well reported and data from 3D cultures highlight the importance of the tridimensionality in drug sensitiveness. The sensitivity of 3D cell cultures for anticancer drugs has been correlated to in vivo animal models data, revealing its ability to bridge the gap between 2D cultures and in vivo models, and potentially to replace preclinical animal models.

Despite the efforts to transfer the 3D tumor models into drug screening platforms for preclinical assays, many challenges remain to be addressed regarding the implementation of high-throughput and high-content screening techniques. The lack of improvement of automated imaging systems for 3D scaffold visualization along with scalability, reproducibility of biomimetic matrices, compatibility with currently available biomolecular assays and detection methods are still a concern. Even through these limitations, technological advances have been achieved, supporting the growing interest in these platforms in preclinical studies.

4. Biomimetic Approaches to Modeling Bone Tumor Microenvironment

Over the last years, many studies have reported new biomimetic materials used to mimic the cell microenvironment and understand the previously discussed ECM–cell interaction, solubility, factor availability, structural and mechanical properties of ECM, and its biodegradability. (Figure 2). These properties, in conjugation with biocompatibility, surface type (sponge, hydrogel, electrospun fibers, and decellularized tissues or monolayers), pore size, and material source (natural and synthetic), have a key role in the modulation of cell shape, cell–cell communication, nutrient uptake, and gene expression. This allow researchers to find out the most suitable biomaterial that reflects the uniqueness of each tissue and pathology in a 3D model.

Bone is a vascularized tissue that continuously undergoes highly dynamic resorption and reformation processes. Its mechanical properties vary from cancellous (2–20 MPa) to cortical (100–200 MPa) bone depending on the interconnected porosity (30–90% in cancellous bone and 10–30% in cortical bone) formed by the main organic and inorganic matrix components, collagen and hydroxyapatite. This complex tissue structure hinders the development of an “ideal bone scaffold” in which the interconnected pores should have micro and macro porosities (pore size < 20 μm and > 100 μm) and the resorption rate should be controlled.

The traditional metallic and bioceramic materials have been extensively used for medical implants for stiff tissues, like bone. With regard to bone engineering, the intrinsic osteoconductive and osteoinductive properties and the similar composition concerning the mineral content have potentiated the integration of bioceramic materials in bone host tissue. However, its inherent brittleness and decline of mechanical properties due to inappropriate resorption rates are limitations faced in bone grafts. Polymeric materials have emerged as a complement to overcome these limitations, being used as a coating on highly porous bioeramic scaffold surface in order to improve its mechanical properties and biological activity through functionalization with growth factors. Further improvements at structural level have been achieved through the implementation of the 3D printing technology, which allow the fabrication of hierarchical structures that closely mimic tissue structure.

In the field of tumor microenvironment modeling, polymeric-based scaffolds have received much more attention than bioeramics materials. These biomimetic materials can be made of
natural-derived polymers, such as collagen and solubilized basement membrane preparation extracted from the EHS mouse sarcoma (e.g., Matrigel), synthetic polymers, including PEG and PCL, or combinations of both types. Engineered tumor models have been disclosed for several tissues and cancer types aiming to recapitulate some features of native tumor microenvironment. In the bone, osteoblasts, osteoclasts, osteocytes, MSCs endothelial and immune cells are part of its cellular milieu. Additionally, bone homeostasis is maintained by low pH, hypoxia, high levels of extracellular calcium and mechanical stimuli. Three dimensional polymeric scaffolds provide essential structural and mechanical support for cells; however, the total recapitulation of the biological complexity is a huge challenge. In this sense, researchers have been working on reachable models of lower level of complexity that can recapitulate the essential features to address a specific question of the tumor under study. Hydrogels, sponges and electrospun fibers are the most studied scaffold types, although decellularized tissues also appear to be promising.[16] The functionalization availability, design flexibility, and biodegradability through molecular design or enzymatic degradation allow their application in a wide range of tissue stiffness. In bone tumor modeling, polymer functionalization with bioceramic materials such as hydroxyapatite (HAp) and β-tricalcium phosphate (β-TCP) has allowed a more faithful recapitulation of the inorganic composition of human bone.[50,51] Although polymeric-based scaffolds are largely applied due to their own advantages, some drawbacks are recognized and have delayed a larger progression on this field of tumor modeling for biomedical research.[17]

Hydrogels are a complex network of hydrated polymeric materials with a water content of around 90% by weight. Besides their high water content, hydrogels are bioactive and, due to their highly tunable biochemical and biophysical properties, can offer structural features similar to native ECM, directly regulating cell fate and function.[52] Hydrogel properties are determined from parameters influenced by the cross-linking density, including volumetric swelling ratio, mesh size of the network, and gel mechanics (e.g., measured by Young’s moduli), as reviewed by Callari and Burdick.[53] Some materials to be applied as hydrogels are already in the market, like PureCol, Matrigel, QGel, Biogelx, and Corgel BioHydrogel. However, all of them exhibit noteworthy drawbacks that affect the cellular behavior and the reproducibility of the 3D models.[53] Spongy-like hydrogels, or sponges, are scaffolds based on a precursor hydrogel which, after freezing, is subjected to freeze-drying and rehydration. During this processing, these scaffolds acquire higher pore size compared to original hydrogels that provide a large surface area to cell adhesion.[54] The physical properties of the hydrogels are improved and the sponge may show additional flexibility and long-term stability. These properties demonstrate that sponges
are useful for 3D cancer modeling, what has been highlighted by many studies, in which have been reported enhanced cell–ECM interaction and more accurate drug screening.[16]

Although there is great progress regarding the application of biomaterials as scaffolds for support cell culture, scaffold-free 3D culture is an extensively explored model in which cells are suspended in culture media, devoid of ECM or scaffold, in order to aggregate and form the named multicellular tissue spheroids (MCTS). These spheroids can be generated through ultra-low attachment plates, hanging drop or magnetic levitation, as reviewed by Nath et al.[19] The possibility of generating a large number of spheroids in the same platform allows high-throughput combinatorial tests.[20] The 3D structure of spheroids allows the recapitulation of cell–cell and cell–ECM biochemical interactions and native gene and protein expression profiles verified in vivo.[21] Due to the faithful mimicking of crucial tumor features and microenvironment, these classic 3D MCTS are considered a standard microtissue for drug screening and have been widely applied in sarcoma research.[22,23]

5. The Current Status of 3D Bone Cancer Models

Despite the great advances in 3D cell cultures, it is essential to search for more biomaterials that accurately mimic the natural ECM, promote cellular growth and, at the same time, provide reproducibility, scalability, and reduced costs. This section describes the state of the art of biomimetic materials applied in 3D osteosarcoma models, how each platform contributes to an in-depth comprehension of cancer growth, and its applicability for drug screening and validation. Some studies regarding the development of bone regeneration and mineralization models are also discussed concerning its contribution in understanding histopathology of bone. Table 1 summarizes the studies developed in the past 10 years in 3D osteosarcoma (OS) models, including the therapy used to evaluate the model’s feasibility to drug development and screening.

5.1. Scaffold-Free Models: Spheroids

Multicellular tissue spheroids (MCTS) are powerful 3D models that have been contributed to our knowledge of pathophysiology of the tissues, being currently considered the most relevant 3D platform in cancer research.[24] Spheroids are characterized by distinct zones with different cellular proliferations and metabolic activities, ranging from a central hypoxic core of necrotic or apoptotic cells to an outer layer of nutrient-rich proliferating cells, with an increasing gradient of metabolites, oxygen, and pH.[25,26] This heterogeneity of MCTS was demonstrated by Rimann et al. through histological characterization of spheroids consisting of different OS cell lines (SaOS-2, HOS, and MG-63).[27] In this study, the authors proved the straight association between the physical barrier provided by microtissue morphology and drug resistance to several anticancer drugs exposure (doxorubicin, cisplatin, taurodilin, pemetrexed, and taxol). Patient-derived OS cells were also tested and the results showed to be consistent with patients’ chemotherapeutic outcome, highlighting the potential of spheroid cultures for preclinical drug screening applied in personalized medicine.

Baek et al. developed a similar study including real-time monitoring of ATP generation and morphological changes as a result of cisplatin cytotoxicity on different tumor spheroid types.[28] Although the cytotoxicity is confirmed by ATP blocking, the maintenance of spheroid structure demonstrates that chemotherapeutic treatments have a crucial effect in ECM-related gene and protein expression, which modulates cell–cell interactions and tumor networks.[29,30] Effectively, Bai et al. evidenced that upregulation of genes involved in OS ECM reconstitution (e.g., collagen, fibronectin) and cell–cell adhesion are directly correlated to chemo- and radiotherapy responsiveness, potentiating a resistant phenotype.[31] In fact, a broad proteomic analysis displayed a high number of species associated with doxorubicin resistance, with a focus on cathepsin D, lately implicated in several malignant phenotypes.[32] These findings explain the less sensitivity of a 3D culture or in vivo assays to clinical chemotherapeutic agents, when compared to conventional 2D cell cultures.

Spheroids of CSCs have been studied in several cancer types and their involvement in disease resistance and tumor relapses have also been hypothesized for OS. Therefore, isolation and characterization of OS-CSCs as well as evaluation of their responsiveness to conventional therapies has been reported. Martins-Neves et al. isolated CSCs from the human MNNG/HOS cell line through the formation of spherospheres in poly-hydroxyethylmethacrylate-coated plates and characterized them regarding their stem-like properties: i) self-renewal, ii) MSC surface markers, iii) pluripotency markers, iv) ATP-binding cassette (ABC) transporters, and v) tumorigenicity.[33] Comparing with the parental lineage, CSCs were less susceptible to apoptosis due to the increased ABC transporters expression and transition to a quiescent state and relatively more resistant to chemotherapy. Additionally, CSCs spherospheres exhibit enhanced tumorigenicity potential, generating larger tumors in vivo.[34,35]

Besides the standard clinically used therapeutic agents, new ones needed to be assessed and MCTS is seen as a “gold model” for this purpose. For example, León et al. reported, for the first time, the cytotoxic effect of an oxidovanadium (IV) complex with the flavonoid chrysin (VOchrys) on MG-63 multicellular spheroids.[36] The decreased viability and changes in shape and volume of the spheroids revealed the sensitivity of OS cells to VOchrys, which was confirmed through tumor growth inhibition on an in vivo xenograft OS mice model.

Due to their role in tumor maintenance, CSCs are a potential target to eradicate tumor chemoresistance. In contrast to traditional chemotherapy, microRNAs (miRNAs) have been identified as potential biomarkers, therapeutic agents, and as tumor suppressors. miRNAs are small noncoding RNA molecules that regulate a wide range of biological processes and their misregulation is associated to cancer. A recent study demonstrated that miRNA-355, already reported as related with OS, is downregulated in OS-CSCs compared to their parental lineage and anti-miRNA-355 treatment in OS cell lines resulted in an increased resistance and stem-like properties expression.[37] In vivo studies demonstrated that pre-miRNA-355 can be combined with traditional chemotherapeutic, improving their outcome.
Table 1. Three dimensional in vitro scaffold-free models for osteosarcoma and bone metastasis research.

| Biomaterial | Scaffold type | Osteosarcoma/bone-derived cells | Co-cultured cells | Therapeutics | Ref. |
|-------------|--------------|--------------------------------|-------------------|--------------|-----|
| Scaffold-free models: Spheroids | | | | | |
| - | - | SaOS-2, HOS, MG-63 | - | Doxorubicin, cisplatin, tauroolidine, pemetrexed, taxol | [58] |
| U2OS | - | - | HOS, HuO9, MG-63, MNNG-HOS, 143B, HS-Os-1, NOS-1, NOS-10 | Doxorubicin, gemcitabine, docetaxel, X-ray irradiation | [61] |
| HOS51 | - | - | SaOS-2, SJSA-1, KHOS/NP, HOS, HuO9, MG-63, MNNG-HOS, 143B, HS-Os-1, NOS-1, NOS-10 | Doxorubicin | [62] |
| MNNG-HOS | - | - | SaOS-2, SJSA-1, KHOS/NP, HOS, HuO9, MG-63, MNNG-HOS, 143B, HS-Os-1, NOS-1, NOS-10 | Doxorubicin, cisplatin, methotrexate, X-ray irradiation | [63] |
| MG-63 | - | - | MG-63, U2OS, 143B | Doxorubicin | [64] |
| MG-63, U2OS, 143B | - | - | MG-63, U2OS, 143B | Doxorubicin | [65] |
| MG-63 | - | U2OS | HUVECs | Doxorubicin | [66] |
| SaOS-2 | - | - | Doxorubicin | [67] |
| Scaffold-based models | Natural Biomaterials | | | | |
| Matrigel | Hydrogel | HOS, 143B | - | pLKO.1 shsFRP2 vector | [68] |
| 143B, MG-63, U2OS, hFOB1.19 | - | - | Paris polyphylla ethanol extract | [69] |
| MG-63 | - | - | miR-29b-1 overexpression | [70] |
| K7M2 | - | - | Bromodomain inhibitor JQ1 | [71] |
| Collagen Type I | Hydrogel | U2OS | - | Kinase inhibitor PI103 | [72] |
| LM8 | - | - | Tyrosin phosphorylation inhibitor (ACM-14) | [73] |
| Matrigel/Collagen Sponge | hFOB 1.19 (osteoblasts) | PC3, LNCaP | siRNA-based gene knockdown | [74] |
| Hydrogel | MOS, U2OS, 143B, ZK58, KPD, SaOS-2 | - | Trametsinib, AZD8330, and TAK-733 kinase inhibitors | [75] |
| Silk Fibroin Sponge | 143.98.2, SaOS-2, U2OS | Bone marrow fibroblasts, HUVECs | Doxorubicin, cisplatin | [76] |
| MOS, U2OS, 143B, ZK58, KPD | - | - | MAB208 (anti-IL-8), Avastin (anti-VEGF-A) | [77] |
| MG-63 | MDA-MB-231, MSCs | - | Paclitaxel | [78] |
| MG-63 | MDA-MB-231 | - | Paclitaxel | [79] |
| Chitosan Sponge | MG-63, MNNG | - | Doxorubicin | [80] |
| Alginate Hydrogel | MG-63 | - | Tetracycline hydrochloride | [81] |
| Fibers | MG-63 | HUVECs | - | NF-κB decoy oligodeoxy-nucleotide | [82] |
| Spheres | Dunn, LM8 | - | - | [83] |
| Synthetic Biomaterials Poly(ε-caprolactone) Eletrospunscaffold | TC-71 (Ewing sarcoma) | - | Doxorubicin, MK-0646 | [84] |
| Porous scaffold | MG-63 | - | Doxemethasone | [85] |
| Poly(ethylene glycol) Hydrogel | Human osteoblasts | LNCaP | - | [86] |

A homotypic spheroid structure acquires hypoxic phenotype, similar to in vivo avascular tumors. However, tumors are heterotypic microtissues and the involvement of endothelial cells and other stromal cells stimulate tumor angiogenesis and enhance tumor growth. The importance of the vascularization process was demonstrated by Chaddad et al. where 3D spheroids of MG-63 cells were deposited on a 2D human umbilical vein endothelial cells (HUVECs) monolayer. This co-culture promoted the formation of a well-organized network of tubule-like structures of HUVECs, stimulated by the enhanced expression of angiogenic factors like VEGF, intercellular adhesion molecule 1, and C-X-C chemokine receptor type 4 as a result of the crosstalk.
between them (Figure 3). In turn, the neo-vascular network expressed higher levels of cluster of differentiation (CD) 31 and collagen IV, specific markers of vascular formation and infiltration. The in vitro co-culture of stromal cells, such as fibroblasts and bone marrow (BM)-MSCs, as well as the epithelial cells in the bone, osteoblasts, that mimic the in vivo situation, demonstrated to have a crucial effect in tumor behavior and drug sensitivity.\[68\] This could be explained by the similarity to the native ECM and cell–cell interaction that act as barriers to the penetration of chemotherapeutic drugs into the tumor. Therefore, it is imperative to develop more complex and reliable in vitro OS models that can more accurately mimic the in vivo cell function and provide physiologically relevant results.

The great results in spheroid-based research field had motivated the development of high-throughput platforms for spheroid formation and, by this way, opened up the possibility to apply these cellular aggregates in preclinical drug screening.\[56,69–71\] Superhydrophobic surfaces patterned with wettable spots proposed by Oliveira et al. are a great example of the effort that have been made in recent years, demonstrating that this robust platform allow the formation of SaOS-2 and L929 cell spheroids and in situ combinatorial high-throughput doxorubicin screening.\[15\] More recently, a new high-throughput, high-content drug screening platform was custom-designed toward the development of 3D tumor invasion models associated with automated imaging and analysis.\[70\] Although pancreatic cancer cell lines had been used to the proof-of-concept, this system has the potential to be applied to other tumor types. Shen et al. also proposed a high-throughput system for phenotypic screening of several pediatric cancer cell lines, including osteosarcoma-derived cells.\[21\] A large collection of approved or under investigation compounds were tested at multiple concentrations on 3D tumor spheroids and many chemical agents were identified as attractive candidates to be used in preclinical models, revealing the applicability of this platform to screen new compounds for pediatric solid tumors treatment.

5.2. Scaffold-Based Models

5.2.1. Natural Biomaterials

Reconstituted Basement Membrane: Reconstituted basement membrane (rBM) of murine EHS sarcoma is a complex protein mixture that contains several ECM proteins (e.g., laminin, collagen type IV, heparan sulfate proteoglycan), endogenous growth factors (e.g., TGF-β, FGF, epidermal growth factor, PDGF), and other proteins and glycosylated molecules that constitute the environment of the tumor.\[72\] Its protein-rich composition, specially the most abundant protein, laminin, promote epithelial, endothelial, and tumor cells attachment, supporting differentiation of some cell types.\[72\] Corning Matrigel is the most recognized commercially available rBM that has been commonly used as a thin layer in 2D cultures and as a hydrogel for 3D scaffold-based cancer models in vitro.

Signaling cascades associated with cancer cells survival and proliferation have been attractive in cancer research owing to the possibility of identifying new targeted therapeutic molecules. Techavichit et al. used Matrigel to assess the role of secreted frizzled-related protein 2 (sFRP2) in OS invasion and metastasis.\[73\] sFRP2 is involved in Wingless signaling pathway and its overexpression has been associated with metastatic ability of cancer cells.\[74\] Therefore, the behavior of OS cell lines with gain or loss-of-function of sFRP2 was compared and, although proliferation was not affected, the metastatic ability was diminished with the sFRP2 knockdown, both in vitro and in vivo.\[73\] Yao et al. also proposed an antimetastatic therapy based on Paris polyphylla ethanol extract, which demonstrated to suppress cell proliferation and inhibit cell migration, invasion, and vasculogenic mimicry formation, in both 3D Matrigel in vitro model and in a xenograft model.\[75\]

Similar to scaffold-free models, scaffold-based ones are used to study CSCs resistance mechanisms and search for new therapeutic agents. An example is the report of Di Fiore et al. where
the authors propose miRNA-29b-1 overexpression as a new therapeutic agent due to its role in enhancing the sensitivity of OS cells to chemotherapeutic drugs and suppressing stemness properties of OS-CSCs.[76] rBM-based hydrogels were also applied in spheroid invasion models to evaluate the pharmacodynamic effect of medronate (BP)-functionalized HAp nanoparticles loaded with the bromodomain inhibitor JQ1 on OS cancer cells.[77] The 2D assays demonstrated a promising selectivity of HAp/BP/JQ1 nanoparticles toward cancer cells, having no toxic effect on cell viability and migration of primary fibroblasts. Regarding OS cell invasion, fabricated nanoparticles also significantly inhibited OS spheroid invasion. Overall, these results demonstrated that this drug-delivery approach could help achieve a multifold increase in the efficacy of JQ1 delivered from HAp nanoparticle compared to JQ1 delivered alone, once it facilitates drug uptake, sustains its release, and hampers its premature metabolization (Figure 4).

Matrigel is widely accepted as a biologically active support for mammalian cell culture, still its poor mechanical properties and in vitro stability have stimulated its combination with other materials.[78,79] In 2016, Stock et al. published a very complete comparative analysis of 2D and 3D tumor models combining several culture conditions: i) culture format (2D, spheroids, microencapsulation into alginate, growth in bioreactors, and embedding into a hydrogel); ii) cancer cell type; iii) hydrogel constitution (Matrigel, collagen, or a mix of both); iv) mono- or stromal co-culture; and v) chemotherapeutic drug.[80] Cell proliferation was observed in all embedded models but the effect of stromal co-culture was partially obfuscated in Matrigel models. In drug treatment assays, the Matrigel growth factors also obfuscated the protective role of fibroblasts. Taking these evidences, Matrigel alone is probably not the better choice for drug screening tests in mono- or co-cultures of invasive models, what was clearly evidenced in a study reported by Nguyen-Ngoc et al. using cancer and normal epithelial breast cells.[81] In another study, three MEK-ERK mitogen-activated protein kinase inhibitors (Trametinib, AZD8330, and TAK-733) were screened in Matrigel-collagen I embedded OS cells and the decreased cell viability indicates MEKI/2 inhibition as a potential treatment approach for OS, validating the results obtained in 2D.[79] Indeed, a phase I clinical trial (NCT02124772) is ongoing to evaluate the efficacy of trametinib in combination with
Implanted into a rat calvaria. The authors found that Aven silencing leads to G2 cell-cycle arrest and Aven-controlled “ataxia telangiectasia mutated” - checkpoint kinase 1 targeting is required to prevent cancer cells from arresting, sensitizing them to standard chemotherapy.

There are several examples similar to the above described in which the survival and invasive potential of various cancer cell types, mainly of breast cancer, is mimicked in 3D Matrigel models, not only to evaluate the contribution of some signaling molecules in the spread of cancer cells throughout the ECM matrix but also to test clinically used chemotherapeutic drugs in order to validate such model as a platform for drug screening. Indeed, bone matrix–based particles seeded with MSCs was or more flexible as verified in cartilage and tendon. In bone, mineralization, collagenous tissues can be more rigid like bone or matrixes to study bone metastasis.

Decellularized Bone ECM: Biomimetic scaffolds of ECM proteins have guided the progress of tissue engineering. However, the composition and architecture of these scaffolds do not fully reconstitute the in vivo ECM and thus the native cellular function cannot be strictly recapitulated. Hence, 3D decellularized matrices emerged as the 3D scaffold that resemble in vivo ECM composition and structure as closely as possible and, despite the difficulties related to decellularization process, they have been applied in several tissues. Bone grafts have been explored to bone regeneration induced by osteogenic differentiation of MSCs in vitro and in vivo. The ability of MSCs to differentiate into osteoblast-like cells without osteogenic media highlighted the osteoinductive properties of the decellularized bone matrix. Furthermore, bone matrix–based particles seeded with MSCs was showed to be an effective support for new bone formation when implanted into a rat calvaria.

Metastasis development toward bone is dependent of the attachment of cancer cells to proteins presented in bone ECM. As bone grafts are an integral recapitulation of bone ECM and promote osteoactivity of seeded cells, cancer cells behavior can be faithfully mimicked in these constructs. Reichert et al. used decellularized matrix secreted from primary human osteoblasts to recreate a 3D bone-prostate cancer metastasis model. Seeded prostate cancer cells strongly adhered to the mineralized matrix and epithelial-mesenchymal transition was verified through gene expression analysis. Signaling pathways associated with increased migration and invasiveness, Ca2+ signaling, and osteolytic bone matrix properties were dysregulated, showing the potential of this bone-derived matrices to study bone metastasis.

Collagen: Collagen is the most abundant structural protein in the native ECM of mammalian tissues and a key protein during ECM remodeling in disease development. Depending on its mineralization, collagenous tissues can be more rigid like bone or more flexible as verified in cartilage and tendon. In bone, collagen comprises about 90% of its total matrix and, in a bone tumor microenvironment, this fibrillar protein is the main component of the ECM, being an attractive biopolymer for tissue engineering applications and in vitro disease models. Type I collagen, typically derived from rodent tail or bovine dermis can be easily engineered to hold the adequate mechanical properties and has been commonly used in 3D cancer modeling. Marine-derived collagen could also be an alternative source to engineering tissues.

Phosphatidylinositol 3 kinase (PI3K)/AKT and mammalian target of rapamycin (mTOR) pathway proteins are overexpressed in some cancer types and, due to their effect in cell growth and migration, they can be potential targets for anticancer drugs. It was in this scope that Fallica et al. developed a 3D model with type I collagen to evaluate the influence of the 3D matrix in cell signaling of U2OS human osteosarcoma, in comparison to 2D culture. As expected, cell proliferation and migration was significantly slower in 3D ECM like matrices and shown to be related with the reduced activation of PI3K/AKT and mTOR pathways. The cellular inhibition of these pathways through kinase inhibitor PI103 was also evaluated, demonstrating that the drug resistance and subsequent poor patient survival is associated to tumor ECM and its mechanical properties. In another study, the effect of the proteolytic enzyme MMP-14 in tumor progression was assessed through the inhibition of tyrosine phosphorylation with a non-phosphorizable cell-permeable peptide, ALCM-14. Various types of cancers were evaluated in 2D and 3D collagen type I matrices, including osteosarcoma (MG-63 cell line) and fibrosarcoma (HT-1080 cell line) bone cancers. Nyalendo et al. found that ALCM-14 induced reduction of tumor cell proliferation within 3D collagen hydrogels, contrary to 2D culture, where no effect was verified. This study evidences once again that there are several mechanisms dependent on the surrounding 3D tumor microenvironment.

MCTS have been widely studied as a scaffold-free platform, as previously discussed. However, in tumors, the biomechanical, biophysical, and biochemical cues of the surrounding ECM have a crucial role in cell behavior and tissue homeostasis. Therefore, the embedding and manipulation of cancer cell spheroids within a polymeric matrix has been a focus to study tumor growth, invasion, and migration also to evaluate drug response. Charoen et al. produced U2OS osteosarcoma and MDA-MB-231 breast adenocarcinoma spheroids and embedded them into matrices of different type I collagen contents. The results show that tumor growth is highly dependent of matrix stiffness and each tumor type has its optimal mechanical environment, wherein stiffer and softer environment are preferred by bone and breast cancer cells, respectively. The treatment of breast cancer MCTS with paclitaxel shows the potential applicability of these models to mimic the pharmacokinetics in vivo, what cannot be achieved in 2D cultures.

The effect of the 3D ECM in the hindrance of chemotherapeutic drugs used in clinic, as doxorubicin and cisplatin, has been explored in order to explain their therapeutic inefficacy. The advances in nanotechnology have fostered the development of nanocarriers to passive or active drug targeting but their poor penetration within the tumor environment has been a challenge. Surface functionalized nanoparticles with proteolytic enzymes are an interesting strategy recently tested in an OS model embedded in a 3D collagen matrix. The particularity of this work rests on the pH-responsive collagenase coating that only suffer hydrolysis in acidic tumor environment, exposing the enzymes that will improve the deep penetration of the nanoparticle. This new approach is paving the way toward nanomedicine evolution for clinical applications.

The recapitulation of the critical event of lung metastasis, transendothelial migration, was successfully achieved by Tanaka...
et al. using the mouse with highly metastatic OS LM8 cell line.\[96\] Their observations revealed that a disruption of endothelial barrier is correlated with the overexpression of VEGF by tumor cells. In fact, VEGF signal inhibition with pazopanib decreased the transendothelial migration in vitro and clearly reduced the size of pulmonary metastasis in vivo, indicating the potential of anti-VEGF therapy as a lung metastasis suppressor. Recently, Fitzgerald et al. focused in the development of a 3D model based on collagen sponges to recapitate prostate cancer–bone metastasis.\[50,97\] The authors developed a collagen scaffold incorporating GAGs to support cell adhesion and nano-HAp to more faithfully mimic the inorganic composition of human bone, improve the osteoconductive and osteoinductive nature of the scaffold and, therefore, enhance the invasiveness capacity of prostate cancer cells.\[99\] This scaffold supported proliferation of prostate cancer cells and osteoblasts, suggesting to be adequate to investigate the transfection ability of gene-based delivery vehicles for small interfering RNA (siRNA) therapy.\[50,97\] The knockdown of the siRNA targeting the RelA gene subunit of the Nuclear Factor κB (NF-κB) transcription factor, involved in bone metastasis, was assessed using siRNA complexed with cyclodextrin but its efficiency was considerably low.\[97\] Nevertheless, this platform was demonstrated to be promising for the development of new siRNA-based gene delivery therapies for bone metastasis through targeted nanoparticles.

Microfluidic devices have aroused a great interest in metastasis mechanisms research due to the possibility of studying complex processes by easily controlling gradients of biochemical and biophysical signals.\[98\] Bersini et al. developed a microfluidic device using soft lithography to study breast cancer–bone metastasis, recapitulating the cancer cells extravasation and behavior within a bone-like matrix.\[99\] The bone microenvironment was mimicked embedding osteo-differentiated hBM-MSCs into collagen type I and lined with a vessel-like channel coated with a monolayer of endothelial cells. The extravasation and transmigration events were recapitated through the introduction of MDA-MB-231 breast cancer cells into the vessel-like channel, generating micrometastasis. The authors demonstrated the involvement of CXCR2 and CXCL5 in these events, revealing the potential of this microfluidic system to study cancer biology and be used as a drug screening platform.

Silk Fibroin: Silk is commonly isolated from cocoons of Bombyx mori silkworms and consists of two proteins: fibroin structural protein and sericins, proteins that hold the fibroin fibers together.\[100\] Silk fibroin has been extensively used in textile industry and its biomedical applications have been emerging in recent decades.\[101\] This biomaterial can be crafted into several morphologies, including microparticles, films, hydrogels, and porous scaffolds (sponges), each one with different mechanical properties, allowing a great variety of applications.\[100\] Its remarkable mechanical strength, toughness, thermal and chemical stability, biocompatibility, biodegradability, and availability has driven its application in tissue engineering, particularly in the form of sponges in bone and cartilage modeling or regeneration.\[100,102,103\]

Structural and mechanical properties of biomaterials depend on fabrication method, which needs to be properly chosen based on posterior application. For bone tissue engineering applications, bone-related protein production (e.g., osteopontin and bone sialoprotein) and calcium deposition are the main biochemical cues to be achieved, which highly depends on cell interaction with the surrounding matrix. This was demonstrated in a study from Correia et al., using human adipose-derived stem cells (hASCs) to evaluate the effects of silk scaffold fabrication on bone tissue formation.\[104\] Taking decellularized trabecular bone as “gold standard” in this work, two fabrication strategies based on different solvents (aqueous and hexafluoro-2-propanol) and structures (lamellar and spherical pores) were compared considering the pore size, bone matrix production, and calcification. The hexafluoro-2-propanol-derived silk fibroin porous scaffold with 400–600 μm of pore size was the one that more faithfully recapitated the osteogenic response of hASCs. Even though natural silk fibroin has outstanding biological properties, some researchers have been working on the improvement of its bioactive properties for bone regeneration applications.\[105,106\]

The potential of silk in bone tissue engineering highlighted the search for new sources (e.g., Antheraea mylitta silkworms) and its application in bone tumor modeling. For example, Tan et al. demonstrated that silk fibroin sponges allow the proliferation of 143.98.2 OS cells as well as the expression of angiogenic-related growth factors similar to what is observed in vivo.\[107\] These results showed that the most significant changes in cellular activity are an effect of the tridimensionality of cell microenvironment when compared to 2D cell cultures. In a subsequent report, the same 3D models were explored to evaluate the chemosensitivity of three OS cell lines (143.98.2, SaOS-2, and U2OS) to doxorubicin and cisplatin, cell cycle specific, and nonspecific cytotoxic drugs, respectively.\[108\] The results showed that cells grown in 3D scaffolds were less sensitive to doxorubicin exposure than cells in a monolayer, which can be explained by the reduced number of cells cultured into 3D models arrested in G1 phase (Figure 5C). However, cisplatin-treated cells showed a similar sensitivity due to its cell cycle–dependent action. These chemosensitivity differences are largely dependent of scaffold structure and properties, confirming that 3D microenvironment and surrounding stroma directly affect cellular response.\[108,109\] As more recently reported by Tan et al., stromal fibroblasts seem to have a positive role in angiogenesis process, promoting the upregulation of related factors like IL-8 and VEGF-A.\[109\] An antiangiogenic treatment with monoclonal antibodies for IL-8 and VEGF-A demonstrated that, although there are efforts to develop anti-VEGF-A therapies, targeted inhibition of CXC chemokines like IL-8 is probably a better approach, since it greatly reduced HUVECs migration in the described 3D model. So, the reported 3D silk models accurately reproduced the drug resistance verified in vivo, evidencing its potential to be applied as high-throughput drug screening systems similar to the above discussed, not only to target tumor monocultures but also to study new therapies in co-cultures.

The successful results with silk scaffolds have aroused the interest to develop co-cultures applied to lung metastasis process recapitulation. At the same time, the design of a platform for specific delivery of anticancer drugs has also been a focus, particularly in the nanomedicine field. In a recent study, the conjugation of these two areas resulted in a 3D silk scaffold to mimic breast cancer metastasis to bone and evaluate target drug delivery.\[110\] Folate conjugated fibroin nanoparticles loaded with doxorubicin were fabricated and its efficiency was evaluated in a co-culture of MG-63 osteoblast-like cells with MDA-MD-231 human
Figure 5. Engineering of breast-bone cancer metastasis on a 3D silk scaffold. A) Direct and indirect co-culture of human breast cancer cells (HBCC, red) and human osteoblast-like cells (HOLC, green) showing a–c) an increase of HBCC population in comparison with HOLC and d–f) the migration of HBCC toward bone-like construct on (a,d) day 1, (b,e) day 3, and (c,f) day 5. B) Cell viability analysis on drug-treated and untreated mono- and co-cultures. Reproduced with permission.[111] Copyright 2013, Wiley. C) Protective role of 3D environment on doxorubicin response in U2OS culture. Reproduced with permission. [108] Copyright 2016, Wiley.

adenocarcinoma cell line. The targeted drug delivery decreased the metastatic cell population, while the normal metabolic activity of osteoblasts and bone mineralization remained, demonstrating that this 3D platform is suitable as a high-throughput screening system for cancer drug discovery and development. In another study, Talukdar et al. reported the use of natural silk scaffolds as a breast-bone cancer metastasis model.[111] The main difference with the above-mentioned study is the construction of a co-culture to more faithfully recapitulate the migration of breast cancer cells to bone microenvironment. In this indirect co-culture, breast cancer cells were able to chemotactically migrate through silk scaffold toward bone-like construct, against the gravity. Metastatic cells showed to chemotactically invade bone microenvironment, where an increase of its population, in contrast with the decrease of osteoblasts population, was observed. Moreover, cancer cells showed an osteolytic effect on bone mineralization through the reduced calcium deposition in co-cultures, which reflects its influence in the deregulation of bone homeostasis. The posterior addition of MSCs to breast cancer cells revealed the highly discussed modulatory role of stromal cells in tumor progression, invasiveness, and angiogenicity, demonstrating its importance for advancing 3D in vitro platforms for therapy development.[112] In this study, an anticancer drug for breast cancer, paclitaxel, was tested in the co-culture 3D model showing a significant increase in drug resistance when compared with breast cancer monoculture[111] (Figure 5A,B). This supports the idea that 3D in vitro co-culture models are a better platform to study and target cancer progression. Silk fibroin-based scaffolds have demonstrated high potential to bone and bone diseased tissue engineering, providing platforms to high-throughput drug screening, discovery, and development as suitable alternatives to the extensively used animal models and overcome 2D model drawbacks. The use of genetically engineered silk proteins can be also an excellent alternative to the natural fibroin, as other functionalities could be added to the macromolecules to tune the properties of the developed tissues.[113]

Alginate: Alginate, a polysaccharide mainly extracted from brown seaweeds, has been widely applied in cell and drug encapsulation.[114] Once alginate lacks cell adhesion motifs required for tissue engineering purposes, its bioactive properties have been improved through the combination with other natural and synthetic polymers, including gelatin and nano-HAp, bioactive particles or grafting adhesive proteins or peptides.[115–118] The cross-linking of HAp into alginate-based hydrogel combined with gelatin microspheres encapsulating tetracycline hydrochloride improved the mechanical features of the scaffold in a manner that potentiate its application in bone tissue regeneration.[115] Besides to sustain the natural behavior of MG-63 osteoblast-like cells, this hydrogel was able to release tetracycline hydrochloride encapsulated into the microspheres, offering a promising
scaffold for bone regeneration and drug delivery in situ. The combination of gelatin methacryloyl with alginate was also used to explore the application of capillary-based microfluidic technology in bone tissue recapitulation. The mechanical properties of this composite material allowed the fabrication of a biomimetic osteon-like structure through a controllable double-layer hollow microfiber, replicating the interaction between endothelial cells that constitute the vascular vessel and bone-derived cells.

Arginine-glycine-aspartate (RGD)-modified alginate hydrogel was used to produce microspheres co-encapsulating anti-bone morphogenetic protein (BMP)-2 monoclonal antibody to induce osteo-differentiation of human BM-MSCs through BMP receptors activation. BMP signals actually seem to be a promising strategy for stem cell-mediated local bone regeneration, herein evidenced through in vivo repair of critical-size calvarial defects. Another strategy based on the same approach was reported by Madl et al., in which murine osteoblasts and MSCs fate were directly controlled by incorporating BMP-2 mimicking peptide to alginate chains, promoting the upregulation of osteopontin expression and increasing mineral deposition. Although these are great results, osteoblast communication with endothelial cells is crucial for bone tissue engineering as demonstrated by Correia et al. through in vitro and in vivo assays.

As described so far, alginate-based 3D cultures have been widely applied in the field of bone regeneration strategies, without exploring its translation for bone disease models. Nonetheless, Akeda et al. revealed the ability of alginate beads to support OS spheroid culture preserving the malignant potential of the cells to metastasize to lung. The promising results obtained both in vitro and in vivo potentiated a further study to elucidate about the specific role of NF-kB in the regulation of pulmonary metastasis of osteosarcoma. The successful inhibition of this transcription factor with a synthetic double-stranded oligodeoxynucleotide shows that, although it does not affect tumor growth, it significantly reduces pulmonary metastasis in vivo, proposing a new antimetastatic target.

Other Natural Biomaterials: In addition to the above-mentioned materials of natural origin, others have been explored in bone tissue engineering, including fibrin, hyaluronic acid, agarose, carrageenan, chitosan, gellan gum, bacterial cellulose, and gelatin.

Polymers involved in crucial biological processes or related with tissues integrity and homeostasis are of huge interest in biomedical engineering. For example, fibrin is an important regulator of wound healing process, constituting the major protein component of blood clots. Its biological activity as angiogenesis inducer has led to its combination with other biopolymers for bone tissue engineering. The formation of fibrin networks into a collagen scaffold showed that it enhanced osteoblast-like cells attachment and proliferation. However, improvements at mechanical strength level need to be investigated in order to better mimic the physical properties of bone matrix.

Hyaluronic acid is another biomaterial naturally abundant in the ECM of connective tissues that, besides the lack of cell adhesion motifs, has been largely applied in the engineering of tissues whose cells express hyaluronan receptors. Effectively, MG-63 cells, positive for CD44, exhibit better proliferation into an interpenetrating double network of this glycosaminoglycan with fibrin than within a fibrin network. Pan et al. studied the metastasis of renal cell carcinoma to bone in hyaluronic acid hydrogels, demonstrating the ability of this biomaterial to support cancer cell proliferation and spheroid formation. Gene expression analysis revealed that the cells maintained their metastatic phenotype into the hyaluronic 3D models, particularly through the highest levels of expression of adhesion molecules, angiogenic and osteolytic factors, contrary to those observed in 2D cultures. The encapsulation of prostate cancer cells in this bone-like environment also highlighted the use of 3D culture systems to reproduce typical cell behavior, as well as the sensitivity to anticancer drugs such as camptothecin, docetaxel, and rapamycin.

Agarose, a polysaccharide derived from the cell wall of some red seaweeds, has been mainly studied for cartilage repair through cell encapsulation. Although this material is not biodegradable and lacks in adhesion motifs, agarose is able to support cell proliferation, including that of human SaOS-2 osteoblasts, particularly in a hydrogel enriched with nanocrystalline carbonated HAp. In the same study, Cabasas et al. included bovine serum albumin as a model protein for drug-release studies and concluded that drug release is dependent on its inclusion strategy and scaffold composition, which can be easily tailored in 3D scaffolds. This polysaccharide also demonstrated to improve the hardness of an alginate–gelatin hydrogel combined with calcium salt of polyphosphate, overcoming the problems of the nonproliferating state of cells in the initial hydrogel and increasing its mineralization.

Carrageenan is another polysaccharide obtained from the same group of red seaweeds that, depending on its sulfate groups composition, can form mechanically different hydrogels. Its similarity with natural GAGs and strong binding to protein, such as the aforementioned silk fibroin, seems to be an advantage for bone tissue engineering, providing a better biomimetic environment to support pre-osteoblast differentiation and mineralization. Moreover, its natural biomechanical properties, particularly its ability as gelling agent, aroused interest in the development of controlled drug-delivery systems through carrageenan-based hydrogel beads incorporating molecules such as growth factors involved in bone tissue angiogenesis.

Gellan gum is a bacterial exopolysaccharide that also resembles ECM GAGs and has been explored for tissue engineering applications in the form of a hydrogel. The combination of methacrylated gellan gum with type I collagen resulted into a hydrogel with remarkable mechanotransduction properties, able to induce the self-osteogenic differentiation of hASCs, offering a promising bone injectable biomaterial.

Gorgun et al. explored the use of bacterial cellulose, a fibrous polymer synthesized by bacterium Acetobacter xylanum to develop a 3D model that aimed to understand the effects of hypoxia on OS stem cells, isolated from SaOS-2 based on CD133 surface protein expression. The ability of this tumor model to investigate the stemness of OS-CSCs was evidenced; however, further improvements of the scaffold may be useful for new therapeutic approaches.

Chitosan-based scaffolds have also been used in tissue engineering due to its similar structure with GAGs, one of the major components of tissue ECM. Bioactive composites based on chitosan may be used to culture osteoblastic-like cells. In
order to develop a model to eradicate tumor recurrence from a tumor-related bone defect, Yang et al. conjugated chitosan with doxorubicin-loaded calcium silicate microspheres and M-type ferrite particles (SrFe\(_{12}\)O\(_{19}\)) to produce a multifunctional magnetic mesoporous scaffold combining chemo-photothermal synergetic therapy with enhanced bone regeneration. In vitro and in vivo assays demonstrated that near infrared irradiation promoted the increase of local temperature and allowed fast doxorubicin release, contributing to a significant decrease of tumor volume while stimulating human BM-MSCs proliferation and, consequently, improved bone regeneration. The in situ release of doxorubicin is a great advantage to minimize the side effects of chemotherapeutic drugs.

5.2.2. Synthetic Biomaterials

*Poly(t-Caprolactone)*: The main advantage of synthetic materials is their easily controllable properties and low batch to batch variability. Poly(t-caprolactone) (PCL) is a common synthetic polymer that has been extensively applied in tissue engineering and drug delivery. Owing to PCL’s biocompatibility, biodegradability, low melting temperature, and tunable mechanical and structural properties, various PCL constructs have been fabricated to recapitulate human tissue according to the intended disease model and case-of-study. This biologically inert polymer has been explored to develop and validate 3D platforms for tumor biology study and drug screening in order to produce in vivo drug sensitivity. Fong et al. used TC-71 cells to engineer an in vitro Ewing sarcoma model in a 3D PCL scaffold produced by electrospinning. Cultured cells acquired the natural phenotype found in vivo, revealing that this porous scaffold is able to mimic biochemical and morphological features, growth kinetics, and protein expression of human tumors. The electrospun PCL scaffold showed an in vivo–like drug sensitivity to insulin-like growth factor-1 receptor and mTOR signaling pathway inhibitors as well as to doxorubicin, validating this platform for combinatorial therapies. These remarkable results obtained for an Ewing sarcoma model can potentially be translated into an osteosarcoma model. Nevertheless, PCL bioactivity is limited and its functionalization with minerals or proteins has been studied to better recapitulate native bone tumor and enhance cell attachment and proliferation. PCL scaffolds are frequently fabricated as fibrous structures applying the popular method used by Fong et al., electrospinning, which produces scaffolds with large porosity but small pores. Fused deposition molding, supercritical compression molding, freeze drying or gelation, centrifugal and melt-blown spinning are alternative methods that have been applied for tissue engineering. The scaffolds were functionalized with an interesting natural source of cytokines and growth factor, the blood platelets, to stimulate cell adhesion, proliferation, and osteogenic activity of osteosarcoma MG-63 cells. The enhanced metabolic activity compared to non-functionalized PCL scaffolds demonstrated the important role of platelet proteins in bone tissue engineering.

A pre-metastatic bone marrow niche was also reproduced in PCL scaffolds coated with calcium phosphate to promote osteogenic differentiation of human MSCs in vitro and bone formation in vivo, in order to demonstrate the potential role of 3D scaffolds in the improvement of preclinical in vivo studies. In this study, the melt-electrospinning technology was used to fabricate the PCL scaffolds, a method that overcomes the uncontrollable deposition of the fibers generated through the classic electrospinning. Therefore, this method allows a higher versatility on design and fabrication of the fibers, with controllable micropatterning and mechanical features. Holzapfel et al. confirmed the development of a functional bone marrow niche, essential for the physiological mimicking of bone microenvironment. The transplantation of this bone construct into immuno-incompetent mice provided a humanized niche for homing highly metastatic prostate cancer cells intracardially injected into the animal model, demonstrating that the biology of macro-metastasis development can be studied in this model as well as therapeutic effects of targeted drugs like denosumab (Figure 6).

In addition to the study of tumor development or metastasis biology, bone regeneration has also been a focus, as is the case of other aforementioned biomaterials. Namely, the development of a model for therapeutic reparation of OS tumor surgical resection defect was studied using PCL scaffolds incorporating dexamethasone. The authors suggest that the PCL model can provide sustained delivery of dexamethasone as a cell-based therapy for bone regeneration. PCL can also be combined with natural polymers, such as chitosan, to produce more versatile biomaterials for tissue engineering. It is expected that such hybrid systems could finally increase applicability in OS models.

*Poly(Ethylene Glycol)*: PEG is a nondegradable synthetic polymer which differs from the other ones for its high hydrophilicity and outstanding biocompatibility, which makes it a popular biomaterial for biomedical applications. PEG network crosslinking density is easily manipulated in order to adapt the mechanical stiffness of PEG hydrogels to different applications. Like PCL, the bioactivity of PEG can be improved by controlled functionalization. Incorporation of cell-binding sites (e.g., RGD peptide) and/or MMP-degradable regions have been successful approaches applied in PEG hydrogels to enhance cell–matrix interactions. PEG diacrylate hydrogels were used to explore the effect of matrix stiffness on the growth and marker expression of CSCs from different tissues (breast and gastric adenocarcinoma, colorectal carcinoma, and OS). Jabbari et al. confined cell encapsulation into micropatterned gels with multiple patterns in order to study tumorsphere average size dependence on CSC markers expression. Moreover, matrix stiffness has shown to play a crucial role on the expression levels of Yes-associated protein and its transcriptional co-activator with PDZ-binding motif, involved in CSC niche regulation, with U2OS cells exhibiting CSC phenotype at a higher optimum matrix modulus (50 kPa), which is in line with the rigidity of its original tissue. As well-reported, this technology of micropatterning has been broadly used to evaluate its ability in modeling cell shape and dynamics, contributing to the understanding of collective cell migration mechanisms.

Sieh et al. established a 3D prostate cancer-bone metastasis model to study the paracrine interactions between a prostate...
cancer cell line, LNCaP, and human osteoblasts.[51] The researchers developed two constructs: LNCaP cell embedded in a PEG hydrogel and a hard tissue-engineered bone of medial grade polycaprolactone-tricalcium phosphate (mPCL-TCP) with seeded primary human osteoblasts. Indirect co-culture through the two constructs promote an upregulation of bone markers, skeletal and vasculature associated genes as well as a significant activation of TGF-β1 in LNCaP multicellular masses. Androgen-responsive genes were also modulated, demonstrating that osteoblast induces osteomimicry in LNCaP cells which is relevant for its metastatic colonization of bone. This 3D in vitro model had demonstrated to recapitulate prostate cancer-bone crosstalk, providing a suitable model for studying mechanisms of targeted therapies in this field.

PEG has also been used to study the paracrine interactions and invasion mechanisms that trigger the tumor metastasis from breast to bone microenvironment and, by this way, provide effective tolls to discover new therapeutic agents.[158,159] Stereolithography-based 3D printing technology has been applied for this purpose once it allows the fabrication of scalable and reproducible layer-by-layer porous scaffolds with precisely controlled architecture through computer-aided pre-designed constructs.[158–160] Zhu et al. fabricated a 3D printed nanocomposite of PEG and HAp nanoparticles with different geometries and applied the most suitable bone matrix for breast cancer cells proliferation in a 3D model to study their interaction with osteoblasts.[158,159] Using a Transwell system with the 3D printed scaffolds, the authors found that breast cancer cells directly affect the proliferation rate and cytokine secretion of osteoblasts. In turn, breast cancer cells growth was stimulated, validating the applicability of this model to explore the biological processes involved in bone metastasis.

6. Challenges and Future Directions

In an era where the increasing number of cancer cases is a daily concern, the development of new therapies is extremely crucial. Tumor growth, invasion, metastasis, and drug resistance is highly dependent on the biochemical and biophysical cues of its own microenvironment. Therefore, the development of 3D platforms that accurately reproduce the dynamic tumor microenvironment and cellular behavior are essential to break the current in vitro disease model’s impasse and this way overcome the lack of predictivity of the most applied preclinical models: 2D in vitro monolayer culture and in vivo animal models. As herein reviewed, a great variety of platforms for 3D OS modeling have been developed using natural, synthetic, or hybrid biomaterials in order to continuously improve the discovery and screening of anticancer drugs. Cancer cell adhesion, proliferation, and invasion have been achieved in such models although chemical functionalization has shown to be essential to improve the biological response, particularly in synthetic polymer-based biomaterials. Despite the need for further evaluation, some osteosarcoma-based studies already demonstrated that drug sensitivity using 3D models is close to the data from animal models, revealing the potential of such 3D models as preclinical platforms for drug screening.

Even though the discussed cancer models offer a great contribution to the understanding of the fundamental role played by the tridimensionality of the cell microenvironment, many questions remain to be answered. The potential impact of crucial parameters such as oxygen, nutrients, and drug concentration gradients, as well as the intimate interaction with tumor-associated stromal cells should be further elucidated. Regarding the drug toxicity assessment, studies need to be performed co-culturing tumor cells with epithelial and stromal cells of the tissue under study in order to predict the in vivo cell response. Additionally, the entire cellular events that underlie the cancer spread and metastasis processes remain broadly simplified, particularly the influence of fluid shear stress which is the major drawback of 3D systems over animal models. So, more sophisticated platforms applying microfluidic devices and bioreactors need to be addressed to recapitulate the in vivo fluid dynamics and mechanical stress. Conversely, 3D models can strictly reproduce the human pathophysiology in terms of totally human-derived components regarding the scaffold materials, culture media, and cells, being promising toward a new era of personalized medicine.

Figure 6. Humanized tissue engineered bone for human prostate cancer metastasis study. A) Fabricated a) tubular scaffolds support b) osteoblast-like morphology of seeded cells (F-actin in red and nuclei in blue). B) Development of in vivo macro-metastasis of human PC3 cells, including to bones where C) the a) implanted tissue engineered bone showed morphological and structural similarities to b) femora of control mice. Reproduced with permission.[146] Copyright 2014, Elsevier.
Advancing therapeutic options through 3D in vitro models, biomaterials, drug screening, osteosarcoma, tissue engineering.

**Keywords**

The authors declare no conflict of interest.

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**Conflict of Interest**

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