Osteosarcoma tumor microenvironment: the key for the successful development of biologically relevant 3D in vitro models

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Abstract

Osteosarcoma (OS) is the most common primary bone cancer in children and young adults. This type of cancer is characterized by a high mortality rate, especially for patients with resistant lung metastases. Given its low incidence, high genetic heterogeneity, the lack of effective targets, and poor availability of relevant in vitro and in vivo models to study the tumor progression and the metastatic cascade, the pathophysiology of OS is still poorly understood and the translation of novel drugs into the market has become stagnant. Due to the importance of the tumor microenvironment (TME) in the development of metastases and the growing interest in targeting TME-specific pathways for novel therapeutics in cancer, models that closely represent these interactions are crucial for a better understanding of cancer-related events. In OS research, most studies rely on oversimplified two-dimensional (2D) assays and complex animal models that do not faithfully recapitulate OS development and progression. In turn, three-dimensional (3D) models are able to mimic not only the physical 3D environment in which cancer cells grow but also involve interactions with the TME, including its extracellular matrix, and thus are promising tools for drug screening studies. In this review, the existing and innovative OS in vitro 3D models are highlighted, focusing on how the TME is crucial to develop effective platforms for OS tumor and metastasis modeling in a physiologically relevant context.

Keywords Osteosarcoma · Tumor microenvironment · Metastasis · 3D in vitro models · Microfluidics

Introduction

Sarcomas are a group of solid neoplasms that originate from mesenchymal cells. These types of cancer are far less common than epithelial-derived carcinomas. In particular, osteosarcoma (OS) is a high-grade primary skeletal malignancy characterized by immature osteoid matrix deposition from cancer cells [1]. It has an annual incidence rate of around 3.1 cases per million people in the USA and 0.3 cases per 100,000 people in Europe, accounting for less than 1% of all newly diagnosed cancer in adults [2]. OS is the most common primary bone malignancy in children and the third most common cancer in adolescence, having a peak incidence between the ages of 10 and 14 years and in individuals over 60 years old [2]. OS develops mainly at the metaphysis or lower extremity of long bones, possibly related to puberty and bone growth that occurs around this age [3]. Risk factors associated with the disease include rapid bone turnover, ionizing radiation to the bone, pre-existing bone diseases (Paget's disease), and heritable genetic syndromes that result in predisposition to OS [3]. Nevertheless, OS appears

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sporadically in about 95% of cases [2]. For the past 40 years, OS treatment has involved neoadjuvant or induction combinatory post-operative chemotherapy. The most commonly used drugs include methotrexate, adriamycin (doxorubicin), and platinol (cisplatin)—the so-called MAP therapy that can be combined with ifosfamide [1, 4]. OS survival rate has increased constantly until the late 1980s with a combination of improved surgical techniques (conservative surgery vs. amputation) and the introduction of chemotherapy [1]. Nonetheless, neither tumor resection surgery nor chemotherapy can control the metastatic spread of OS. Moreover, OS is usually radio-resistant and, in 90% of cases, OS metastasizes to the lung, leading to an extremely low 5-year survival rate (<20%) [5]. The low OS prevalence coupled with high tumor heterogeneity between patients and resistance to anti-cancer drugs has greatly hampered the development of effective therapies due to difficulty in finding targetable events. Another hurdle is the complex tumor microenvironment (TME) that is composed of bone cells, immune cells, stromal cells (mesenchymal stem/stromal cells (MSCs) and fibroblasts), vascular cells (endothelial cells and pericytes), and a dense mineralized extracellular matrix (ECM) [6]. Recently, the OS TME has been studied as a target for novel therapies [6, 7]. However, these studies are associated with controversial results and failures in the improvement of the efficacy of treatments, which remains a considerable challenge to overcome in this research field. One possible reason for this is the lack of relevant in vitro platforms that include the TME and allow to study OS progression and its metastatic process.

In OS-related research, two-dimensional (2D) models with immortalized cell lines have been substantially explored for experimental and drug screening studies. Great knowledge has been acquired from these cell culture assays; however, they fail to recapitulate the dynamic environment in which cancer cells grow, including proper cell–cell and cell–ECM interactions that influence cancer progression and drug resistance [8]. Moreover, considering the TME as a relevant disease defining factor brings the value of contemplating biomechanical and physical cues and not only the genetic changes that promote tumor cell proliferation [9]. The introduction of novel three-dimensional (3D) models has changed our ability to investigate cancer in vitro, bridging the gap between oversimplified 2D assays and overly complex animal models that often do not represent human-specific events. 3D models offer multiple features that faithfully mimic the 3D architecture of solid tumors and also their interactions with the surrounding TME, including (i) the ECM as an important player in cancer pathogenesis; (ii) the possibility to culture cells under dynamic conditions, including vasculature-mimicking systems; (iii) and increased controllability regarding specific cell responses (e.g., ECM stiffness, oxygen/metabolic levels,

interpatient heterogeneity), allowing to undercover cancer-related events under a relevant microenvironment [8, 10]. Numerous scaffold-based models have been developed to mimic the ECM in vitro [11]. Biomaterials ranging from Matrigel® (reconstituted basement membrane of murine Engelbreth-Holm-Swarm sarcoma), collagen, or alginate to synthetic alternatives such as poly(lactic acid) (PLA) and poly(ethylene glycol) (PEG) or even decellularized matrices have been thoroughly explored to properly culture cells within a biomimetic environment [12–14]. Other strategies employing tumor spheroids and organoids provide a more complex approach emulating the interactions between multiple cell types and drug penetration profiles while preserving specific tumor features, which is especially important for drug screening studies [15, 16]. Emerging technologies such as 3D bioprinting and microfluidics represent a step forward in cancer modeling in vitro, allowing the creation of complex multicellular systems for high-throughput drug screening with a high level of controllability and physiological relevance [17, 18].

This review presents a brief overview of bone homeostasis, followed by a description of how the bone microenvironment is transformed in OS. Thereafter, the TME impact in lung metastasis formation is discussed as well as how it is possible to target the metastatic cascade and the TME to improve OS treatments. Finally, the available OS 3D in vitro models are presented, focusing on how it is possible to improve them toward the establishment of more relevant culture systems for modeling OS and its metastatic spread in vitro.

Bone homeostasis vs. osteosarcoma pathophysiology

The bone is a highly plastic tissue that constantly changes, growing and remodeling up until the early stages of adulthood. The three main cell types that form the bone tissue include osteoblasts, osteocytes, and osteoclasts. These cells interact with each other and with the surrounding environment to modulate and maintain bone homeostasis. When stimulated to promote bone formation, osteoblasts can inhibit the activity of osteoclasts. This balance between osteoblast and osteoclast activity is essential for bone homeostasis and the constant renewal of old bone and its replacement with newly synthesized mineralized bone matrix (Fig. 1a) [19]. Osteoblasts have parathyroid hormone (PTH) receptors that when bound to PTH lead to the expression of receptor activator of the nuclear factor- κ B (NF- κ B) ligand (RANKL). This ligand binds to its receptor RANK on hematopoietic osteoclast precursors, promoting osteoclast differentiation. Conversely, osteoprotegerin (OPG) produced by osteoblasts and stromal cells is a potent inhibitor of osteoclast

a. Bone homeostasis **b. Tumor-induced bone remodeling**

Fig. 1 Bone homeostasis vs. osteosarcoma-related bone remodeling. **a** Bone homeostasis is a complex process tightly regulated by the RANKL/RANK/OPG signaling pathway. RANKL is secreted by osteoblasts and osteocytes stimulated by PTH, vitamin D3, and prostaglandin E2. RANKL binds to the RANK receptor in the membrane of preosteoclasts differentiated from HSCs due to M-CSF activity, produced by immune cells and osteoblasts. Mature osteoclasts promote bone resorption, which is inhibited by OPG that binds to RANKL, blocking RANK signaling. **b** In OS, bone remodeling is induced by tumor cells that create a vicious cycle to promote cancer cell survival and proliferation. Tumor cells secrete RANKL,

IL-6, IL-11, and TNF- α that stimulate osteoclastogenesis, leading to greater secretion of bone factors that promote tumor cell survival. Moreover, OS cells release factors, such as TGF- β , that promote the secretion of RANKL by osteoblasts and inhibit their activity, decreasing osteoblastic bone formation. Tumor cells also promote the secretion of sclerostin (Scl) from osteocytes that inhibits osteoblast's activity. This creates a tumor-enhancing environment where the expression of OPG is generally decreased, while the expression of RANKL is normally enhanced. PTHrP, PTH-related protein, SEM4D, semaphorin-4D

differentiation, inhibiting the RANK/RANKL interaction [20]. PTH also controls OPG secretion to maintain bone homeostasis [20]. Osteoclast activity is also promoted by tumor necrosis factor (TNF)- α and macrophage colony-stimulating factor (M-CSF) produced by immune cells and osteoblasts, whereas transforming growth factor (TGF)- β produced by macrophages can induce the recruitment of MSCs, which are osteoblast precursors, thus leading to osteoblast proliferation [21]. In addition, RANKL reverse signaling is also an important pathway regulating bone homeostasis, in which RANK secreted from extracellular vesicles (EVs) of maturing osteoclasts binds to osteoblastic RANKL, thus promoting bone formation [22].

OS oncogenesis and its metastatic spread are currently poorly understood. The molecular characterization of OS also remains incomplete mainly due to high intratumoral and intertumoral heterogeneity, the epigenetic complexity of the disease, and its low incidence. Although the origin of OS is still debated, evidence suggests that MSCs or MSC-derived pre-osteoblasts may acquire genetic alterations that lead to OS formation and proliferation under the influence of specific TME signals [23]. Given that OSs have many characteristics of immature osteoblasts, more committed osteoblastic precursors could also be a part of OS origin.

Effectively, some data support OS originating from a more committed osteoblast pool of cells rather than MSCs [23]. Osteogenic differentiation is a tightly regulated process as a result of multiple signaling pathways (WNT and bone morphogenetic protein (BMP)), transcriptional regulators (TP53, RUNX2, ZEB1, and ZNF521), and cell cycle controllers (RB1) [24, 25]. Therefore, genetic alterations (such as germline mutations in TP53, RB1, RECQL4) may result in incompletely differentiated osteoblasts or osteoclasts that disrupt the balance between proliferation and differentiation of bone cells, thus leading to uncontrolled cell proliferation that may slowly expand to originate bone tumors [24]. Moreover, osteoclast activity is unregulated in OS, leading to excessive bone resorption (Fig. 1b). Tumor cells secrete RANKL, interleukin (IL)-6, IL-11, and TNF- α , which stimulate osteoclast activity, thus leading to increased release of bone matrix entrapped factors (such as platelet-derived growth factor (PDGF), insulin-like growth factor (IGF)-1, and TGF- β) that promote cancer cell survival and proliferation [26].

The accumulation of these genetic alterations has been reported to generate a subpopulation of OS cancer stem cells (CSCs) able to self-renew and sustain tumor progression through interactions with the TME [27]. This cancer stem

cell niche is also associated with drug-resistance events related to increased expression of drug efflux systems (ABC transporters and Aldefluor activity), altered DNA repair activity, epigenetic factors, and expression of anti-apoptotic proteins [28]. Moreover, conventional chemotherapy has been shown to induce the selection of stem-like cells in OS, specifically through the activation of the WNT/ β -catenin signaling pathway [28, 29]. Therefore, targeted therapies have been explored to tackle this issue, including inactivating the Hedgehog, Notch, and WNT signaling pathways [27, 30]. For instance, the early steps of metastasis, such as invasion and intravasation of tumor cells, are promoted by the activation of the WNT/ β -catenin pathway [31]. This signaling pathway induces the acquisition of stem cell properties by OS cells; promotes ECM remodeling, allowing tumor cell invasion; induces the expression of vascular endothelial growth factor (VEGF), leading to angiogenesis; and enhances the formation of an immune tolerant environment by enhancing pro-tumorigenic macrophage polarization and inhibiting the activity of cytotoxic T cells [31]. Inhibiting the WNT signaling pathways has been demonstrated to prevent OS metastasis in a human xenograft model on immunodeficient mice [32]. However, these signaling pathways are related to skeletal development in children, so it may be unsafe to inhibit them [3].

Other relevant pathways implicated in OS pathophysiology include phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR), RANKL/NF- κ B, TGF- β , and IGF signaling pathways [3, 33]. All these signaling pathways have been explored to develop novel OS treatments. Unfortunately, disappointing results from recent clinical studies highlight the need for novel targets. For instance, the combination of sorafenib, a small molecular inhibitor of multiple tyrosine kinase receptors (RAF, KIT, VEGFR, PDGFR), and a rapamycin analog that inhibits the mTOR pathway showed promising results in preclinical studies but a recent clinical trial revealed only a 45% progression-free survival rate [34]. Therefore, there is a clear need for novel targets and more robust preclinical models to faithfully recapitulate OS progression both in vitro and in vivo in order to understand relevant cancer-TME interactions that lead to OS development and metastases.

How can the tumor microenvironment influence metastasis?

The metastatic spread of OS accounts for the poor survival rate associated with the disease. The metastatic cascade comprises multiple steps from cancer cell invasion of the surrounding tissue, intravasation into blood/lymphatic vessels, survival in circulation, extravasation into the secondary organ, and establishment of a secondary tumor in a distant

site [35]. Since the lung is an efficient organ for filtration, OS cells preferentially metastasize to the lung due to mechanical restriction. The human alveolar capillaries have a smaller average diameter than OS cells that favors their entrapment within the lung tissue [36]. Another possible explanation is the development of a pre-metastatic niche (PMN). This concept encompasses the creation of a fertile environment in a secondary organ for the establishment and proliferation of metastases. The importance of the PMN in OS has been recently demonstrated in an orthotopic xenograft model [37]. OS cells were demonstrated to release TGF- β -expressing EVs that promoted the production of high levels of IL-6 from MSCs, thus stimulating the activation of intratumor STAT3 (cell proliferation-related transcription factor), which in turn led to tumor growth, enhanced invasion potential, and lung metastasis formation [37]. Additionally, another study revealed the activation of lung fibroblasts to a cancer-associated phenotype by OS cell-derived EVs [38]. This process was shown to be dependent on TGF- β 1 expression by OS EVs, highlighting how tumor cells can modulate the lung PMN to support the metastatic process [38, 39]. Furthermore, OS cells not only control the formation of a favorable distant PMN but also interact with other non-neoplastic cells, supporting the creation of a pro-metastatic TME in the bone.

Cell crosstalk within the osteosarcoma tumor microenvironment

The OS TME is a highly plastic and complex environment composed of multiple cell types (mainly bone, stromal, and vascular cells) immersed in a dense mineralized ECM that surrounds and communicates with cancer cells [6]. The crosstalk between all these cells facilitates immune evasion by tumor cells, angiogenesis, and cancer cell intravasation and dissemination, as well as the dysregulation of the bone remodeling process (Fig. 2). Moreover, these complex and dynamic interactions, together with high tumor heterogeneity and a lack of knowledge of its specific signaling pathways, thwart the development of effective therapies, especially for highly resistant metastatic OS tumors.

Osteoblasts, osteoclasts, and cancer cells

As mentioned, the molecular OPG/RANK/RANKL triad is crucial for the maintenance of the bone niche. Once OS develops in the bone, tumor cells secrete factors (PTH-related protein, IL-11, TGF- β 1) that initiate osteoclast-mediated bone reabsorption, a characteristic of most osteolytic bone tumors [40]. The RANK receptor and RANKL are normally expressed at the surface of osteoclasts and osteoblasts, respectively, but in the OS TME they can be expressed by tumor and immune cells. While osteoblasts

Fig. 2 Osteosarcoma tumor microenvironment interactions. (a) Tumor cells interact with endothelial cells and MSCs within a hypoxic environment, promoting the release of HIF-1α VEGF, which induces angiogenesis. (b) Bone marrow-derived MSCs are attracted to the tumor site by CXCL12 and MCP-1 secreted by OS cells. Within the TME, MSCs are normally “educated” by tumor cells (through TNF signaling) to have pro-tumoral activity and can differentiate into cancer-associated fibroblasts. Circulating macrophages can infiltrate the TME and differentiate into pro-inflammatory M1-like macrophages that have anti-cancer activity, or anti-inflammatory M2-like macrophages that express RANK and promote tumor

proliferation and angiogenesis. (c) OS cells promote immunosuppression through the activation of T regulatory cells, myeloid-derived suppressor cells, and neutrophils that suppress the activity of antigen-presenting cells and inhibit cytotoxic CD8⁺ T cells. (d) Cancer-associated fibroblasts are activated by FGF and PDGF released by cancer cells, and promote ECM remodeling, inducing the formation of a dense matrix structure, which in turn promotes the formation of a necrotic tumor core, leading to VEGF secretion and angiogenesis. ARG1, arginase 1, iNOS, inducible nitric oxide synthase, LPS, lipopolysaccharide, SDF-1, stromal cell-derived factor-1

and stromal cells typically secrete OPG, in the OS TME, there is a reduced expression of this molecule, leading to an uncontrollable proliferation of osteoclasts [41]. Therefore, high levels of RANKL/RANK in the TME are associated with worse OS outcomes. Moreover, the dysregulation of this cycle leads to increased release of pro-tumorigenic factors trapped in the bone matrix [40]. For

instance, TGF-β is known to enhance cell migration/invasion of tumor cells by the increased production of matrix metalloproteinases (MMPs) and promote osteoclasts’ osteolytic activity by the enhanced expression of RANKL and IL-11 [42]. It also upregulates VEGF and PDGF, growth factors that have a key role in angiogenesis and tumor growth. Finally, it also exerts immunosuppressive effects

by regulating immune cell proliferation and activity [42]. In the OS TME, the vicious cycle between osteoclasts and OS cells is also exacerbated by the release of EVs from tumor cells. In contrast to RANK-bearing EVs released from osteoclasts to promote bone formation, tumor cells can produce EVs with pro-osteoclastic factors. Indeed, EVs containing TGF- β , MMPs, CD-9, and RANKL were isolated from 143b OS cells, thus highlighting a novel communication mechanism between tumor cells and the TME to induce bone destruction [43].

Mesenchymal stem/stromal cells and fibroblasts

MSCs are multipotent non-hematopoietic precursor cells found in many human tissues, primarily in the bone marrow. They can differentiate into multiple lineages (osteoblasts, chondroblasts, myoblasts, and adipocytes) while having the ability to self-renew [44]. MSCs migrate to the OS TME due to a strong tropism of monocyte chemoattractant protein (MCP)-1, growth-regulated oncogene (GRO)- α , and TGF- β 1 [45]. Once in the TME, MSCs exhibit a cancer-associated fibroblast (CAF) phenotype and respond to tumor cells that express TNF- α in hypoxic conditions by increasing the expression of chemokines (CC motif chemokine ligand (CCL)5, CCL2, C-X-C motif chemokine ligand (CXCL)1, CXCL12, IL-6, IL-8), and VEGF that promote cancer cell survival, proliferation, invasion, and transendothelial migration [45–47]. These paracrine and autocrine factors released by MSCs affect various cell types in the TME, including immune, endothelial, and bone cells. For instance, CCL2 and CCL5 are related to monocyte and macrophage recruitment to the TME, while IL-8 mediates the establishment of an immunosuppressive environment, and VEGF promotes endothelial cell proliferation and angiogenesis [44].

Furthermore, in OS, MSCs, CAFs, and osteoblasts secrete a dense ECM, characterized by tight collagen fibers [6]. Consequently, immune cells, such as T cells, have difficulty penetrating the ECM to reach the tumor site, hindering their anti-cancer activity and the efficacy of immunotherapies. MSCs also secrete EVs that allow communication with osteoblasts and OS cells, thus enhancing tumor cell survival and migration [48]. MSC-secreted EVs have been shown to carry OS supportive microRNAs and growth factors, as well as lactate and glutamate, that favor cancer cell proliferation and metabolism [6]. Conversely, OS cells inhibit osteogenic differentiation of MSCs, keeping them in an undifferentiated state through the TGF- β /Smad2/3 pathway, which promotes the secretion of pro-tumorigenic factors by MSCs [49]. This was also demonstrated in a different study where OS cells were shown to release TGF- β rich EVs promoting the secretion of IL-6 by MSCs [37], which in turns leads to enhanced OS migratory potential and stemness [50].

Immune cell crosstalk

The immune microenvironment of OS is not yet fully understood. However, some evidence suggests that it has a significant role in the metastatic spread of cancer cells [51]. The immune niche of OS is mainly composed of tumor-associated macrophages (TAMs). Other immune cells present include dendritic cells and lymphocytes [51]. Macrophages play a crucial role in the bone environment, influencing bone formation (through oncostatin secretion) and acting as osteoclast precursors in the presence of M-CSF and RANKL. Circulating monocytes are normally recruited to the OS TME by cancer cells (through CCL2, CCL5, CSF-1, VEGF, IL-34 secretion) and then may differentiate into macrophages. TAMs may also originate from tissue-resident macrophages and monocyte-related myeloid-derived suppressor cells (MDSCs) [52]. In the OS TME, TAMs possess high plasticity and can adopt multiple phenotypes according to the environmental signals received [53]. Generally, at either end of a broad polarization spectrum, classically activated or M1-polarized macrophages demonstrate anti-cancer activity as they promote the secretion of pro-inflammatory signals (IL-6, IL-12, IL-1, TNF), leading to an increase in immune cell activation. By contrast, alternatively activated or M2 macrophages are known as pro-tumorigenic cells that release anti-inflammatory signals (IL-10 and VEGF) related to immunosuppression, tumor progression, and angiogenesis [53]. In the context of OS, the high proportion of TAMs in the TME has been associated with reduced metastasis and improved survival, although both M1 and M2 macrophages are present [54]. Even though conflicting results have shown a lower survival rate related to higher TAM infiltration [55], studies have demonstrated the detection of more M1-like TAMs in non-metastatic patients, which is associated with the anti-tumor activity of these subtype of macrophages [56]. A higher number of M2-like TAMs suggest the formation of a more immunosuppressive microenvironment, with less proliferation of effector T cells and increased levels of immunosuppressive TIM3⁺PD-1⁺ (T cell immunoglobulin domain and mucin domain 3/programmed cell death protein 1 positive) T cells [51, 56]. Moreover, M2-polarized TAMs communicate with OS cells and MSCs to maintain the stem cell-like phenotype of cancer cells [57], and can release IL-1 β , a pro-inflammatory cytokine that activates the NF- κ B and TNF signaling pathways, which are related to the enhanced tolerance of OS cells to chemotherapy [58].

The establishment of this complex immune niche within the OS TME is orchestrated by tumor cells, capable of controlling the recruitment and differentiation of immune cells, thus creating a tumor-favoring environment to potentiate cancer progression, metastasis, and drug resistance. Indeed, OS cells can dysregulate the balance between M1 and M2 macrophages, which stimulates a switch in the phenotype of

TAMs from M1-like macrophages to pro-metastatic M2-like macrophages [51, 56]. Moreover, OS cells also control the response of T cells via the PD-1 and programmed cell death protein–ligand 1 (PD-L1) recognition system, which inhibits the cytotoxicity activity of T cells and consequently leads to the formation of an immunosuppressive environment ideal for tumor growth [55, 59]. In addition, the relatively low abundance of cytotoxic CD8⁺ T cells in the OS TME may suggest that OS tumors are poorly immunogenic, with a scarce infiltration and activity of lymphocytes [60]. This lower immune infiltrate level hinders the effectiveness of immune checkpoint inhibitors and the possibility to use targeted therapies that rely on immune cell activation.

Vascular microenvironment

The vascular niche not only regulates osteogenesis and hematopoiesis but also promotes tumor irrigation and survival by providing oxygen and nutrients to tumor cells. It also fosters the intravasation of cancer cells into blood vessels and their migration into a specific metastatic location [61]. The hypoxic and acidic bone microenvironment promotes the release of pro-angiogenic factors (hypoxia-inducible factor (HIF) and VEGF) by tumor cells, leading to the formation of new blood vessels to irrigate the tumor site [61]. However, this process of neovascularization in OS remains poorly understood. It has been shown that neovascularization occurs mostly from pre-existing blood vessels, but there is also increasing evidence of the presence and activity of endothelial progenitor cells that can differentiate into mature endothelial cells after recruitment from the bone marrow [6, 62]. Furthermore, the formation of non-endothelial microchannels from OS cells, a process called vasculogenic mimicry, has also been reported and associated with a worse prognosis [63]. OS tumors are highly vascularized tissues, a fact that has been suggested as a poor prognostic factor. Moreover, it has been demonstrated that patients with low OS vascularization have a better survival outcome and a better response to chemotherapy [64]. By contrast, high expression of VEGF and VEGFR is associated with a lower survival rate and it is considered to be an effective biomarker of OS prognosis [65]. The activation of PDGFR by platelets also has been linked to increased OS cell growth and a poor prognosis [66].

Extracellular matrix

The main components of normal bone ECM are significantly altered in OS. ECM production is considerably increased, resulting in a stiffer stroma and a more aggressive cancer phenotype. Not only tumor cells but also activated fibroblasts enhance the deposition of collagen, fibronectin, laminin, and proteoglycans which leads to the formation of

a stiffer and denser ECM [67]. Moreover, ECM remodeling is accomplished by the enzymatic degradation of ECM proteins by MMPs (MMP-2, MMP-9, and MMP-13 that function via the ERK-associated signaling pathways) and heparinase released by tumor cells. This allows cell invasion into adjacent tissues and intravasation into blood vessels [67]. ECM remodeling is also important in the lung, where OS cells usually extravasate to and establish metastases [68]. Recent studies have identified key ECM molecules responsible for OS development that may also be relevant prognostic biomarkers [67]. For instance, OS cells overexpress collagen triple helix repeat containing 1 (CTHRC1), which is related to metastasis development and a poor prognosis due to inhibition of collagen type I deposition [69]. Upregulation of $\alpha v \beta 3$ integrin and fibronectin has also been reported in OS, guiding tumor cell migration [70]. These specific ECM components could be a target of novel therapies to hamper the progression of OS, as it was demonstrated by a recent study [71], thus showing the importance of considering the ECM as a major player in OS progression.

The tumor microenvironment and the metastatic cascade as novel targets for osteosarcoma therapy

The current treatment for OS consists of surgical tumor resection in combination with chemotherapy, including doxorubicin, cisplatin, and methotrexate [72]. This therapy is usually effective for about 50% of patients. However, for patients with metastases or relapsed OS, the survival rate is only around 20%, which emphasizes the urgent need for more effective treatments [5]. Therefore, multiple novel targets are being explored to improve the efficacy of OS therapies [7, 73] (Fig. 3). Given the importance of the RANKL–RANK pathway in bone homeostasis and its dysregulation in OS, studies have investigated the potential of inhibiting this pathway to improve OS outcomes [41]. However, while whole-body deletion of RANKL proteins in a genetically engineered mouse model prevented OS development and metastases, RANK deletion in osteoblasts did not hinder OS progression [74]. Denosumab, a monoclonal antibody against RANKL approved for metastatic breast and prostate cancer, has also been explored for OS and it is currently in a phase II clinical trial [75]. Preclinical results have demonstrated its protective effect against bone loss [75, 76]; however, doxorubicin-inhibiting events have been reported [77]. Since the role of osteoclasts in OS tumorigenesis is still controversial and the RANKL reverse signaling pathway has not been studied in OS, more studies still need to be performed to explore this avenue for OS treatment.

In terms of targeting the bone microenvironment, ongoing studies include the utilization of bisphosphonates that prevent bone loss and have anti-angiogenic properties which support tumor regression [78]. Functionally,

Fig. 3 Targeting the osteosarcoma tumor microenvironment. The strategies to target the OS TME to develop novel therapeutics include the following: (1) targeting TAMs by reprogramming M2-like macrophages into an anti-tumorigenic M1 phenotype, and promoting phagocytosis by inhibiting the SIRPα receptor; (2) targeting the ECM by inhibiting the tumorigenic activity of certain molecules (collagen-IIIα1, αvβ3 integrin); (3) using monoclonal antibodies against immune checkpoint inhibitors (PD-1, PD-L1, CTLA-4, HHLA2, B7-H3) to promote the activity of cytotoxic T cells and inhibit the activity of MDSCs and T regulatory cells; (4) targeting osteoclast

activity (with bisphosphonates), the RANKL/RANK signaling pathway (with monoclonal antibodies), and the activity of the TGF-β receptor; (5) inhibiting the pro-tumorigenic activity of MSCs; (6) targeting multiple tyrosine kinase receptors (VEGFR, PDGFR, FGFR, IGF1R, HER2) that promote tumor cell survival and proliferation, as well as angiogenesis; (7) targeting other cell surface receptors overexpressed in OS cells (GD2) and promoting tumor cell apoptosis by enhancing the activity of the FAS receptor (CD95). LPS, lipopolysaccharide, MHC-I, major histocompatibility complex I, TCR, T cell receptor

bisphosphonates interfere in osteoclast differentiation which hinders bone resorption and also have a direct role in OS cells' proliferation, leading to suppressed tumor growth and decreased expression of VEGFR [79]. The safe inclusion of bisphosphonates in adjuvant chemotherapy for OS has been reported; however, no clinical trials have demonstrated the effectiveness of this therapy [80].

Furthermore, the critical role of the immune system in cancer development and the complex crosstalk between

tumor, immune, and bone cells has paved the way for the development of novel immunotherapies. Given the lack of specific markers of mesenchymal cells and the high heterogeneity of OS tumors, OS-specific antigens have been challenging to identify [3]. Nevertheless, one promising breakthrough has been immunoadjuvant therapy with mifamurtide (muramyl tripeptide phosphatidylethanolamine) [81]. The available MAP therapy is not specific for cancer cells and promotes the killing of effector T cells. Contrariwise,

mifamurtide is a synthetic analog of a bacterial protein that can activate the immune system through the receptor NOD2 (nucleotide-binding oligomerization domain-containing protein 2) highly expressed in monocytes, maintaining a balance between pro-inflammatory M1 macrophages and pro-tumorigenic M2 macrophages [82]. Moreover, mifamurtide adjuvant therapy is related to increased serum levels of pro-inflammatory cytokines that hinder cancer progression. However, its use is controversial and only approved in the European Union [83]. Other promising immunotherapy molecules include type I interferons (IFNs), such as IFN- α which enhances OS cells' sensitivity to chemotherapy, as well as immune checkpoint inhibitors, such as anti-CTLA-4 (cytotoxic T lymphocyte-associated protein 4) and anti-PD1/PDL1 [3]. However, recent clinical trial data has shown that single-agent checkpoint inhibitors therapies do not seem to contribute to tumor regression, which can be partially explained by the low neoantigen burden of most OS tumors [84, 85]. Other alternatives include targeting the immune-checkpoint inhibitors HHLA2 and B7-H3 (CD276), as well as targeting the disialoganglioside GD2 with a monoclonal antibody in combination with granulocyte-M-CSF (GM-CSF) to stimulate the anti-cancer activity of macrophages [7].

Furthermore, multiple studies and clinical trials are being performed to target receptor tyrosine kinases or their ligands overexpressed in the OS TME. Explored targets include VEGF, IGF-1, PDGF, HER2, and MET receptor tyrosine kinases [7, 86]. Unfortunately, the clinical trial results do not seem promising. Single targeting VEGF with the monoclonal antibody bevacizumab to inhibit angiogenesis has demonstrated low efficacy in improving OS outcomes [87]. Moreover, inhibiting IGF-1R has also shown disappointing results in phase II trials with patients with recurrent OS [88]. The most promising strategy has been targeting multiple tyrosine kinase receptors at the same time or combining specific targeting approaches with chemotherapeutic agents. For instance, cabozantinib can simultaneously inhibit VEGFR, PDGFR, MET, KIT, RET, and AXL receptor tyrosine kinases [86]. In addition, its efficacy has been demonstrated in phase II clinical trials, where a progression-free survival of 6.2 months was reached, the longer achieved for all similar treatments tested to date [89].

Lung colonization by cancer cells is associated with multiple metabolic adaptations that could also be possible targets for OS therapy. Notably, a recent epigenetic study has demonstrated considerable differences in enhancer activity between primary and metastatic OS [90]. This variation was also preponderant between metastatic and non-metastatic OS cell lines. The same study also highlighted that the expression of the variant regions is not random and may be positively selected to permit lung colonization by OS cells. One of the genes regulated by these regions—factor

3 (a gene that activates blood clotting)—showed particular importance for metastasis. Moreover, the knockdown of AP-1 transcription factors that occupy these variant regions inhibited cell outgrowth in the lung, thus underlining that these genomic regions have possible targets for OS therapy [90]. Another study highlighted the elimination of cancer cells through the activation of a cell death pathway related to the expression of the FAS receptor (CD95) [91]. Therefore, only FAS-negative OS cells were able to colonize the lung environment. Other targetable metabolic sensitivities associated with OS metastasis include the upregulation of glycoprotein 78, protein kinase C, IL-6, and CXCL8 activity, as well as altered glutathione metabolism [36]. For instance, a recent study has shown that the aberrant expression of Δ Np63 responds to signals from lung epithelial cells [92]. Xenografts, syngeneic mouse models, and canine models were employed in this study, demonstrating higher levels of IL-6 and CXCL8 produced by cancer cells linked to upregulation of Δ Np63. Moreover, a specific combination therapy was able to inhibit the activity of these molecules, leading to inhibition of metastasis formation [92].

Given that most OS patients already appear to have lung metastases at the diagnosis stage, some argue that inhibiting metastatic progression should be the most biologically relevant measure to evaluate the efficacy of novel drugs [93]. However, to boost the translation of therapeutic approaches to the clinic, it is still necessary to improve our understanding of the OS metastatic cascade. For that, the interactions between OS cells and the lung microenvironment must be further explored, as well as the influence of the TME. Finally, improving the available cell culture and in vivo models should also strengthen our ability to faithfully recapitulate the metastatic progression of OS and effectively test and find novel OS targets and treatments.

Current landscape of osteosarcoma 3D in vitro models

In OS-related research, the development of novel preclinical in vitro models remains a challenge, due to the complexity and heterogeneity of OS, which is proven by the lack of relevant in vitro assays to study OS. Most of the available culture models are too simple and do not entirely mimic the TME and the pathophysiology of OS. Consequently, animal models continue to be one of the most used tools for OS studies. However, they are usually expensive, difficult to work with, too complex, and do not faithfully emulate cancer progression, which thwarts the translation of novel drugs into the market [7]. Indeed, more than 90% of novel cancer drugs/therapies do not reach the clinic, which can be partly explained by the poor reliability of our in vitro and in vivo models [94]. Thus, it is of particular importance

to understand how we can improve the current in vitro models to properly investigate the OS metastatic cascade. This section focuses on the available experimental models to study OS, centered on 3D scaffold-based models, spheroids, organoids, and tumor-on-a-chip platforms (Table 1). The strengths and limitations of each model are discussed as well as how it is possible to improve them to further mimic the TME and allow for suitable drug screening studies.

Scaffold-based models

Scaffold-based assays provide a simple matrix for tumor cell seeding. They are defined as biomimetic environments forming a porous ceramic or polymeric structure that allows to emulate the natural environment in which cells grow [12, 115]. More sophisticated models may include sponges,

hydrogels, decellularized matrices, or a combination of materials to originate, for instance, bio-responsive hydrogels to mimic the ECM. The bone tissue has been extensively explored for tissue engineering applications and for investigating cancer metastasis to the bone [116, 117]. This has paved the way for the development of multiple strategies to mimic the bone microenvironment in vitro that stand relevant for further exploitation, namely, to create innovative platforms for OS modeling.

The bone microenvironment is a complex biomechanical system composed of multiple cell types dispersed in a dense and porous matrix. The bone matrix is composed of an inorganic phase (65%) of mostly hydroxyapatite (HA) nanocrystals, an organic phase (25%) of primarily type I collagen, and water (10%) [19]. The HA nanocrystals present in the bone structure promote toughness and strength, while

Table 1 Examples of available osteosarcoma 3D models

3D model	Cell types	Fabrication technique	Scaffold material	Drug screening	Refs
<i>Spheroids</i>	MG63	Hanging drop	–	VO-chrysin	[95]
	MG63, U2OS, 143b	ULA plates	–	Anti-miR-355, cisplatin	[96]
	HOSS1	ULA plates	–	Doxorubicin, gemcitabine, docetaxel, X-ray	[97]
	MG63, 143b, LM7	PHEMA coated plates	–	Doxorubicin, cisplatin, methotrexate	[98]
	SaOS-2	Hanging drop technique in microfluidic chips	–	Doxorubicin	[99]
	MG63	Microfluidic chips with non-adherent surfaces	–	–	[100]
	SaOS-2/MSCs	Agarose microwells of 3D printed molds	–	Doxorubicin	[101]
	MG63, SaOS-2	ULA plates	PLMA, PEGDA, Matrigel®	–	[102]
<i>Hydrogels</i>	MG63 (MSCs/osteoblasts)	ULA plates	PLMA	Doxorubicin	[103]
	MG63	Cell suspension mixed with hydrogel solution	Collagen-I, Matrigel®, agarose, alginate	–	[104]
	U2OS	Cell suspension mixed with collagen solution	Collagen-I	Kinase inhibitor PI103	[105]
	143b, MG63, U2OS, hFOB1.19	Cell suspension mixed with 3% Matrigel®	Matrigel®	–	[106]
	MOS, U2OS, 143b, ZK58, KPD, SaOS-2	Cell suspension mixed with hydrogel solution	Matrigel®/collagen	Trametinib, TKIs	[107]
	MG63 + HUVECs	Microfluidics-based technique	Alginate, GelMA	–	[108]
	Dunn, LM8	Cell encapsulation in alginate beads	Alginate	NF-κB decoy	[109]
	MG63, MNNG	Organic template method	Calcium silicate/chitosan	Doxorubicin	[110]
<i>Porous scaffolds</i>	MG63, SaOS-2	Direct foaming process	Collagen-I/HA nanocrystals	–	[111]
	MG63	Synthetic porous scaffold	PCL	Dexamethasone	[112]
	SaOS-2	Emulsion casting	Water-in-PDMS	–	[113]
	U2OS, fibroblasts, HUVECs	Freeze drying to form porous sponges	Silk fibroin	Avastin, MAB208	[114]

GelMA, gelatin methacrylate; *HA*, hydroxyapatite; *HUVECs*, human umbilical vein endothelial cells; *MAB208*, human IL-8/CXCL8 antibody; *MSCs*, mesenchymal stem/stromal cells; *NF-κB*, nuclear factor-κB; *PDMS*, polydimethylsiloxane; *PEGDA*, poly(ethylene glycol) diacrylate; *PHEMA*, poly(2-hydroxyethyl methacrylate); *PLMA*, methacryloyl platelet lysate; *TKIs*, tyrosine kinase inhibitors; *ULA*, ultra-low attachment

collagen provides elasticity and flexibility. Moreover, bone continuously undergoes a dynamic resorption and formation cycle; thus, it is under constant stress and mechanical stimuli. Therefore, to mimic the bone environment, an ideal scaffold should not only be biocompatible but should also have an adequate pore size (at least 100 μm) to allow the diffusion of molecules and cells; the mechanical properties should match the ones from bone tissues (Young's modulus of cortical bone varies from 15 to 20 GPa); and since high levels of extracellular calcium, hypoxia, and low pH are normal in bone tissues, these properties should also be taken into account when designing biomimetic bone scaffolds [118].

Keeping this in mind, multiple materials have been employed to produce scaffolds to study OS, such as Matrigel®, collagen, alginate, chitosan, silk fibroin, agarose, gelatin, bacterial cellulose, and methylcellulose [119, 120]. Matrigel® is one of the most used materials to perform 3D cell culture. For instance, Matrigel® has been used to fabricate a Matrigel®-collagen matrix to evaluate the potential of MEK-ERK mitogen-activated protein kinase inhibitors as novel drugs for OS treatment [107]. Three cell lines were found to be resistant, while other three were sensitive to the inhibitors, with high constitutive ERK activity (signaling pathway related to cancer cell survival). Nevertheless, Matrigel® has disadvantages for the construction of drug screening models; since it is a material of animal origin, some growth factors can affect the tumor's response to specific compounds and there are problems related to batch-to-batch variations, which limits standardization and reproducible results [121].

Another commonly used material is collagen type I. In the bone microenvironment, collagen type I comprises more than 90% of the organic ECM, playing an important part in OS TME. To recapitulate the stem cell niche of OS, Mg-doped HA and collagen scaffolds were produced by a biomineralization process, allowing the establishment of a proper environment for the maintenance of cancer cell stemness when compared to 2D systems [111]. In a different study, MG63 and 143b cells were embedded in a collagen type I matrix either enriched or not with bone granules [122]. Results demonstrated increased invasion of 143b cellular aggregates from the more complex bone-mimicking matrix to an outer collagen, laminin, and fibronectin hydrogel. Differences in ECM composition also influenced OS cell response to doxorubicin treatment. In the more complex ECM, events of resistance to drug-induced death were observed, which emphasizes the importance of the ECM in the tumor's response to chemotherapeutic agents [122].

Finally, alginate—a commonly used polysaccharide in cell and drug encapsulation approaches—has also been applied to produce bone-mimicking scaffolds. Aiming to exploit alginate's bioactive properties, highly porous

scaffolds were produced by freeze-drying with a combination of alginate, hydroxypropyltrimethyl ammonium chloride chitosan, and oyster shell powder [123]. The scaffolds were biodegradable and biocompatible when tested with OS cell lines and suitable for cell growth and bone-mimicking applications [123]. In a different approach, a highly metastatic cell line was cultured in alginate beads [109]. The in vitro model was used to perform transfection of the murine LM8 cell line with an NF- κ B oligodeoxynucleotide to suppress the activity of the transcription factor, leading to a marked decrease in pulmonary metastasis in the mice injected with transfected cells [109]. In another study, four different scaffolds (collagen type-I, Matrigel®, alginate, and agarose) were used to culture osteoblasts and OS cells [104]. The proliferation rates of cancer cells were higher in more rigid matrices, and an integrin-mediated focal adhesion pathway was identified as a significant player in the sensitivity of MG63 cells to the ECM, which was not verified in 2D flat surfaces [104]. This elasticity-dependent behavior of OS cells in contrast to the adherent-dependent growth of osteoblasts highlights how the mechanical properties of the ECM can influence cancer cell growth, which is a key aspect to consider when applying novel biomaterials in OS tumor modeling. The use of composite materials or nanoenabled 3D matrices are attractive alternatives in the design of biomaterial-based strategies that enable to fine-tune both the composition and the mechanical properties of 3D scaffolds to better resemble the tissue of interest [124, 125].

In this context, given the diverse biophysical environment of the ECM during cancer progression, synthetic biomaterials have been explored to closely tune their features on demand capable of properly mimicking complex dynamic systems. Synthetic biomaterials offer low batch-to-batch variation and the possibility to adjust the properties of the scaffold in a more controllable manner. For instance, the chemical composition, porosity, and stiffness can all be modulated independently to properly mimic the desired environment. Commonly used polymers include synthetic polymers PLA, PEG, polycaprolactone (PCL), and poly(glycolic acid) (PGA) [119, 120]. These materials have mainly been applied to tissue engineering and drug delivery approaches. For instance, a bone-mimicking scaffold was developed to provide a homing spot for cancer cells [126]. PCL scaffolds coated with calcium phosphate promoted osteogenic differentiation of MSCs and the formation of a specialized bone niche for cancer cell migration [126]. For OS, PEG diacrylate hydrogels have been developed to encapsulate U2OS cells [127]. The optimum matrix stiffness for cancer cell growth was evaluated, showing higher optimum stiffness for OS cells when compared to other cancer cell lines [127]. Moreover, PEG has also been applied in bioengineered scaffolds to study interactions between cancer and bone cells. For instance, 3D printed PEG and HA scaffolds

were developed to promote the proliferation and interaction of cancer cells with osteoblasts [128].

In addition, more than being used as a 3D tool for cancer modeling, scaffolds have also been studied as biomaterial-based strategies for OS treatment [129]. For instance, thermosensitive poly(lactic-co-glycolic acid) (PLGA)-PEG hydrogels have been applied to localized co-delivery of doxorubicin, cisplatin, and methotrexate [130]. This strategy was then employed in human OS SaOS-2 xenografts in nude mice, which enhanced tumor suppression and increased tumor cell apoptosis. In another study, which aimed to deliver adriamycin-loaded PLGA nanoparticles to bone tumors, nano-HA/collagen scaffolds were used to encapsulate the nanoparticles, resulting in a better release profile and a significant inhibition of cancer cell growth [131].

Multicellular tumor spheroids

Multicellular tumor spheroids (MCTSs) are one of the most used platforms in cancer research, especially for anti-cancer drug screening. MCTS are able to recapitulate the structure of small solid tumors with cells organized concentrically, mimicking the oxygen, pH, and nutrient gradient observed in tumors [15]. Generally, in the center, there is a lower diffusion of oxygen and nutrients, which leads to a necrotic core formation, whereas more proliferative cells are observed in the outer edges of the spheroids [15, 132]. Multiple techniques can be employed to form MCTS, ranging from the hanging-drop to the liquid-overlay technique based on ultra-low attachment (ULA) plates, or other methods based on magnetic levitation, spinner flasks, matrix encapsulation, microfluidics, and 3D bioprinting [115, 133]. Moreover, easy adaptability to high-throughput screening (HTS) makes spheroids an excellent model for drug diffusion studies and to assess the effectiveness of anti-cancer drugs *in vitro* [133].

In order to evaluate the efficacy of MAP chemotherapy in OS cells, spheroids were formed in non-adherent plates with three different cell lines (143b, MG63, and LM7) [98]. The MG63 and LM7 cell lines exhibited greater resistance to methotrexate when compared to the 143b cell line and MG63 monolayers. Similarly, as a way to test the effect of doxorubicin on the viability of cancer spheroids in real time, U2OS spheroids were established in ULA plates [134]. Results showed higher resistance of OS spheroids when compared to 2D cultures. In addition, since the spheroids were still viable after one day of treatment, cytotoxicity of doxorubicin seemed to happen after endocytosis [134]. OS spheroids have also been used to analyze the role of cancer stem cells in anti-cancer drug resistance. Cancer stem cells were isolated from the MNNG/HOS cell line by culturing OS cells in ULA plates [135]. The tumor cells formed spheroids and after passaging the cells, third-generation spheroids were reintroduced into adherent conditions and allowed

to grow in monolayer. The cancer stem cells demonstrated MSC markers (CD73, CD90, CD13, CD105), self-renewal, and expression of pluripotency properties (differentiation into osteogenic, chondrogenic, and adipogenic lineages). Besides, they were more resistant to chemo and radiotherapy when compared to the parental cell line. In a different study, a double culture spheroid model was developed to study the differences between bone regeneration and chemotherapy in early- and late-stage OS [101]. SaOS-2 cells and MSCs were seeded on non-adherent micromolds to form spheroids with different ratios (MSCs:SaOS-2 3:1, early, and 1:3, late). Early-stage spheroids were generally smaller, produced more ECM, and presented a lower expression of β -catenin, RUNX2, and alkaline phosphatase (ALP), known OS prognostic markers. The addition of PDGF and BMP-2 predominantly affected the early-stage model by inducing a marked increase in size and ECM production, demonstrating that cancer cells were less sensitive to these osteogenic growth factors. Moreover, the combination of growth factors and doxorubicin led to inhibition of the stimulatory effects previously detected, highlighting a limited regeneration capability when chemotherapeutic agents are employed [101].

Furthermore, spheroid-based models have been utilized in combination with hydrogels to perform invasion studies and to better mimic the 3D TME, including the ECM as an essential factor in cancer cell proliferation and anti-cancer drug response. To investigate the influence of cell arrangement in drug screening, a hydrogel model was used to encapsulate OS cells [132]. MG63 spheroids were formed by the liquid overlay technique in ULA plates and encapsulated in either gelatin methacrylate (GelMA) or Matrigel®. The same was performed with randomly dispersed OS cells within the hydrogels. Results showed higher invasion potential and drug resistance in the encapsulated OS spheroids when compared to the cell-laden hydrogels [132]. In a different approach, a human methacryloyl platelet lysate (PLMA) hydrogel was fabricated to study the invasion potential of OS spheroids [103]. This biomimetic material of human origin potentiated the proliferation and invasion of MG63 spheroids that were embedded in PLMA hydrogels and co-cultured with human osteoblasts and bone marrow-derived MSCs. The invasion kinetics could be controlled by variations in the hydrogel stiffness and was higher in the co-culture conditions. Moreover, MSCs migrated toward the OS spheroids and improved tumor cell resistance to doxorubicin treatment, thus showing to accurately recapitulate OS events using an *in vitro* platform suitable for drug screening studies [103]. In another study, MG63 spheroids were cultured over a monolayer of endothelial cells [136]. Rapid tumor cell invasion of the endothelial surface was detected as well as the formation of endothelial cell tubule-like structures that reached the tumor spheroids. Higher expression of angiogenic factors (VEGF, CXCR4, intracellular adhesion

molecule 1 (ICAM1)) was detected, thus confirming the angiogenic potential of cancer cells cultured in contact with endothelial cells [136].

Overall, these studies clearly demonstrate the need for novel spheroid-based OS models. Although some experiments have been done by culturing tumor cells with osteoblasts and MSCs [103] or endothelial cells [136], only one study has reported the establishment of heterotypic MCTS (with cancer cells and MSCs) to model OS in vitro [101]. Thus, the inclusion of stromal cells, such as MSCs, fibroblasts, macrophages, T cells, or bone cells, could be an alternative to develop 3D models that more accurately resemble the OS TME. For other cancer types, multiple studies have reported a successful double culture of spheroids with fibroblasts, MSCs, immune cells, endothelial cells, and other non-malignant cells of the TME [137]. A few experiments have also been performed with triple culture spheroids with tumor cells, fibroblasts, and immune cells (monocytes/macrophages) [138–140]. These studies have shown the potential of exploring more complex MCTS to mimic the TME and that are suitable for drug screening studies in vitro. However, despite their simplicity and HTS capability, similarly to scaffold-based assays, limitations involve the lack of controllability in terms of cell arrangement within the spheroids, which is mostly based on self-aggregation, and thus does not mimic the physiological conditions in some cases. For instance, CAFs usually surround the tumor core and do not penetrate into the spheroid's center, as it has been reported by different studies [138, 141]. Other difficulties arise from the challenge in controlling the number of immune cells or slower proliferating cells within the spheroids' structure, which limits reproducibility and may compromise the ability to test immunotherapies [138].

Tumor organoids

Tumor organoids represent another relevant cancer modeling tool, bringing the advantage of preserving patient-specific tissue characteristics and heterogeneity over multiple passages and recapitulating the native tumor formation in vitro [16]. Tumor organoids are originated from patient/mouse-derived tumor cells or by genetic editing of induced pluripotent stem cells that are cultured on a matrix, normally Matrigel® or collagen [16, 142]. Limitations of this model include difficulty in reaching in vivo-like maturity, time-consuming protocols, and lack of stromal cells and vasculature [143]. Most of the tumor organoid models have been described for epithelial-derived cancers [16]. Nevertheless, a recent study reported the establishment of an organoid culture system of lung metastatic OS tissue [144]. Patient-derived tumor tissues from OS bone or lung metastatic lesions were cultured en bloc in collagen type I hydrogels or as single cells embedded in Matrigel®. The organoids

formed after 7 days and could be propagated for at least 6 months. The OS organoids preserved the morphology of typical human OS tumors and expression of vimentin and transcription factor Sox9. Interestingly, the OS lung organoids retained a similar T cell distribution at earlier passages, which could potentiate their use as an immunotherapy screening platform. Effectively treating organoids with anti-PD-1 monoclonal antibodies led to higher numbers of CD8⁺ T cells, thus attesting T cell activation and faithful recapitulation of the immune TME in vitro. The OS organoids were also cryopreserved, showing no differences in viability, thus suggesting the possibility of creating an OS organoid biobank [144].

Microfluidic platforms

Microfluidic-based 3D models involve microchips with specific designs that gather the advantages of culturing cells within biomimetic transparent microchannels using smaller volumes. This renders microfluidic-based models less expensive than other approaches, and also allows facile imaging analysis as well as HTS adaptability for drug screening studies [18, 115]. Some drawbacks include specialized skills to produce the chips; some technical impairments due to shear stress in the channels, which affect cell proliferation by hindering laminar flow, and few alternative materials to produce the chips as polydimethylsiloxane (PDMS), the most commonly used material, might absorb small molecules [18, 145]. In the case of OS, only a few studies have reported their utilization. These studies mostly focused on the influence of molecular gradients in cell morphology, or the adhesive properties of OS cells under shear flow or at various temperatures/pHs. In one study, the secretion of VEGF type A from MG63 cells was investigated [100]. OS cells were cultured either in a monolayer or in a spheroid model fabricated in microfluidic chips. The results demonstrated lower cell viability under higher stress levels (due to inhibition of HIF and decreased nutrient supply) in both cell culture models. However, in monoculture, the secretion of VEGF decreased in stressful conditions, which does not correspond to the in vivo situation [100]. In another study, a microfluidic platform was developed to test the efficacy of methotrexate and methotrexate-loaded nanoparticles [146]. U2OS cells were seeded inside the microfluidic chips and cytotoxic analysis was performed after 72 h of treatment recirculation. The loaded nanoparticles greatly reduced the cell population to less than 20% for lecithin-polyvinyl alcohol (LEC-PVA) nanoparticles and to 2.3% for LEC-Tween nanoparticles, which was significantly more efficient than the free drug [146].

Furthermore, since microfluidic chips can be adapted for culture under dynamic conditions, they are useful tools to mimic the vasculature in vitro [147] and to study the

metastatic cascade and the migration of tumor cells from the primary tumor [18, 148]. However, no experiments have been conducted to develop an accurate microfluidic platform to unveil the metastatic process of OS cells to the lung. Nonetheless, the lung microenvironment has been extensively studied in organ-on-a-chip platforms [18], which could be explored to analyze the interactions between OS cells and the lung environment. The lung-on-a-chip was one of the first systems developed to include endothelial cells and lung epithelial cells as well as to allow the simulation of breathing motions in vitro [149]. Later, the same platform was adapted for cancer experiments by culturing non-small-lung cancer cells in the device [150]. It was revealed that breathing motions suppressed tumor cell proliferation and invasion, and promoted resistance to tyrosine kinase inhibitor therapy, which appeared to be related to signaling changes of epidermal growth factor receptor (EGFR) and MET protein kinase.

Given that the bone is a common organ for metastasis formation, mainly in patients with metastatic breast and prostate cancer [151], multiple models have been developed to emulate the bone microenvironment in vitro [152]. In one study, fibroblasts, endothelial cells, and tumor cells were co-cultured in a composite gel of HA and fibrin inside a microfluidic chip [153]. Higher concentrations of HA reduced the pore size of the scaffolds, altered their mechanical properties, and reduced cell migration and angiogenic sprout formation. Regarding the extravasation of breast cancer cells into a bone-mimicking environment, another microfluidic platform was utilized to co-culture tumor cells, endothelial cells, human bone marrow-derived MSCs, and osteoblast-differentiated cells embedded in a fibrin gel to recapitulate the bone matrix [154]. The extravasation rates were substantially higher in the bone-mimicking microenvironment, whereas microvasculature permeabilities were higher in myoblast-containing matrices. This demonstrates how distinct microenvironments can influence cancer progression and how this is an important factor to keep in mind when developing cell culture models for drug screening. In another study, a 3D vascularized bone model was established by culturing endothelial cells, bone marrow-derived MSCs, osteoblasts, and osteoclast precursors in a collagen/fibrin hydrogel enriched with calcium phosphate (CaP) nanoparticles [155]. The combination of all these cells and the presence of CaP nanoparticles promoted the differentiation of osteoblasts and osteoclasts compared to monocultures, highlighting the importance of 3D microenvironment composition to accurately recapitulate biological responses in vitro. In summary, the potential of microfluidic-based models to study OS remains an unbeaten path, with full freedom to explore the capabilities of these platforms as powerful drug screening tools and in unveiling unknown processes in OS

progression and metastasis, as it has been investigated for other cancer types.

Modeling metastasis: are 3D in vitro models enough?

Around 90% of cancer deaths are related to metastasis formation even though only around 0.01% of cancer cells can effectively form secondary tumors, which poses the question: how such an inefficient process can be fatal for so many patients? The metastatic cascade involves complex 3D interactions between cancer cells, the secondary organ, and the ECM. Hence, models that can better resemble key aspects of the metastatic cascade are essential for a better understanding of the proliferation of secondary tumors in distant organs. Given the complex metastatic cascade that generates a successful seeding of malignant cancer cells in a distant metastatic niche, it is difficult to study cancer cell dissemination outside of the whole organism. Therefore, animal models are essential for these studies as well as to evaluate the efficacy, safety, and biodistribution of novel therapeutics [156]. An ideal model of OS should allow the spontaneous formation of the primary OS tumor in the bone and the development of lung metastases within an immunocompetent host. Conventional models for studying novel therapies and metastasis most frequently involve xenogeneic and syngeneic mouse models [157]. Currently, there is no robust OS animal model that can completely recapitulate the formation of bone tumors in the same anatomical locations as in humans and with spontaneous development of lung metastases. Xenogeneic models involve the utilization of immunodeficient animals injected with human tumor cell lines, whereas syngeneic models are immunocompetent but involve the utilization of mouse tumor cells and the tumor formation is not spontaneous [157]. Another relevant strategy has been to study the spontaneous formation of OS in dogs, which represent a large population of animals affected by the disease with similar biological behavior, genetic instability, and clinical progression patterns as in humans [158].

The inclusion of complementary systems, such as animal model-related technology, ex vivo tissue approaches, or bioengineered 3D culture models, has recently gained wider appeal. Intravital video microscopy or window for high-resolution imaging in the lung encompasses a small circular window that is implanted in the chest cavity of a live mouse [159]. This assay permits serial imaging of the same area of the lung for up to 2 weeks, which enables to study the establishment of micrometastases. One key disadvantage of this technique is the depth of imaging, which is limited to between 200 and 700 μm depending on the type of microscope used [159]. The pulmonary metastasis assay (PuMA) is an ex vivo model in which lung explants are co-cultured

with fluorescently labeled tumor cells and maintained in *in vitro* culture for up to 21 days. OS cells cultured on this model have been shown to retain the phenotypic features of cancer cells [160]. Indeed, multiple studies have investigated the proliferation of OS cells in *ex vivo* lung tissues and how gene manipulation or drug treatments affect lung metastases [90, 160, 161]. The advantages of this assay encompass the inclusion of a relevant 3D microenvironment to study OS metastasis to the lung and easy cell imaging compatibility by fluorescence microscopy. The major limitation is that some cell lines are not compatible with this model and some observations are only possible for 21 days due to the destruction of lung parenchymal cells over time [36].

Furthermore, novel bioengineered cancer models (spheroids, organoids, tumor-on-a-chip platforms) are crucial breakthroughs in the field of cancer research, allowing for a better recapitulation of tumor-specific events *in vitro* while enabling a relatively simple recreation of the TME and the metastatic process [148, 162, 163]. For instance, in an attempt to merge the benefits of microfluidics and spheroid-based assays, a 3D culture platform has been developed for scale-up production of spheroids in an injection-molded plastic array device [164]. This platform was then used to study tumor angiogenesis by culturing endothelial cells that organized in perfusable blood vessel networks and migrated toward the center of MCTS with fibroblasts and cancer cells. Furthermore, multi-organ-on-a-chip devices have also been explored in the context of metastasis [165]. In one study, a metastasis-on-a-chip model was engineered to assess the preference of cancer cells in terms of metastatic sites [166]. Cancer spheroids were embedded in a synthetic ECM hydrogel and seeded in a compartment of the device. The migration patterns of the circulating cancer cells were evaluated under recirculating fluid flow to either an endothelium, lung, or liver compartment. Cancer cells preferentially migrated to the lung and liver constructs, thus replicating the situation *in vivo* [166]. This rather simple approach highlights the potential of these platforms that have also been thoroughly explored to culture multi organ-like constructs in combination to assess biological responses to drug administration [167].

In terms of metastasis modeling, tumor-on-a-chip approaches present numerous advantages, allowing (i) compartmentalization of cells, thus being possible to introduce multiple cell types in co-culture; (ii) flow and gradient control that allows, for example, the maintenance of endothelial cells in a dynamic culture and the perfusion of particles and immune cells to target cancer metastases; (iii) real-time analysis of cell migration to study cancer cell invasion, extravasation, or intravasation; (iv) control of mechanical forces such as breathing motions or ECM stiffness that may influence cancer cell responses; (v) facile data analysis and imaging through fluorescence confocal imaging [148].

Nonetheless, a complete recapitulation of the complex metastatic process *in vitro* is not yet possible. Reducing production costs as well as specialized and complex equipment (pumps, tubing, etc.) involved the platforms' production, and preserving patient-specific tumor heterogeneity, the TME immune landscape, and the tumor's vasculature is a significant hurdle to overcome to accurately recapitulate the metastatic cascade *in vitro*. Moreover, some chip designs may hinder cell retrieval and the small size of some platforms might impair downstream analysis (e.g., flow cytometry analysis and gene expression studies) since the number of cells may not be sufficient to reach the equipment's sensibility. Recently, 3D bioprinting has greatly contributed to an easy microfluidic chip fabrication process with lower costs, and some pump-free alternatives have also been developed and commercialized, thus highlighting the potential of these emerging technologies to properly culture cells *in vitro* [168–170]. Despite these problems that still need to be tackled, 3D metastasis models have proven to be physiologically relevant and an important tool in the advent of personalized medicine, drug screening, and metastasis studies *in vitro*. In the specific case of OS, none of these models have been explored, which leaves multiple possibilities to investigate their potential for OS *in vitro* modeling and drug screening studies (Fig. 4). Therefore, considering these models in the preclinical pipeline will certainly not only help to reduce the number of animals in cancer research but also expedite the drug development pathway toward a more efficient process with fewer unexpected failures in clinical trials.

Remaining concerns and future perspectives

Over the past decade, 3D culture models have become increasingly available, with multiple companies producing novel and standardized platforms for 3D cell culture and even high-throughput drug screening. However, in the pharmaceutical industry, preclinical research is still primarily based on 2D assays and animal models. To bring 3D models into this setting, these novel platforms need to prove that they are reliable and reproducible systems. Moreover, they must have the capability to be scaled up to an industrial setting to test thousands of compounds in a short time frame. Nevertheless, some challenges still need to be addressed for this to be a reality. For instance, issues related to medium composition, cell sources, and variability/reproducibility still need to be tackled, as different cell types have specific nutrient requirements, implying that the choice of the correct culture medium for the co-culture of multiple cell types may be an additional challenge [171]. Possible solutions include mixed medium, supplemented medium, and partitioned culture conditions [171]. Microfluidic platforms have also shown great potential for culture medium optimization. In

Fig. 4 Framework for the development of novel osteosarcoma bioengineered models. Schematic representation of how innovative 3D models can be employed to study the metastatic spread of OS cells to the lung on a microfluidic chip adaptable for culture under dynamic conditions. OS cells might be cultured on a separate compartment as spheroids or organoids that can be assembled with other cell types to resemble the TME, namely immune cells (macrophages, lymphocytes) and stromal cells (MSCs, fibroblasts, osteoblasts, osteoclasts), on a bone-mimicking environment composed by natural hydrogels (collagen type I, Matrigel®, decellularized matrices (dECM)),

synthetic polymers (PGA, PLA), or composite materials (PLMA, CaP-HA). OS cells growing on this compartment should be able to migrate into the vascular channel with endothelial cells and reach the lung compartment. Importantly, the platform should be adaptable for drug screening studies and to evaluate potential pharmacological targets to inhibit the metastatic spread of OS cells. To further enhance the predictable value, patient-derived tissues could be used, thus harnessing the advantages of personalized medicine and improving the preclinical pipeline before performing animal studies

a recent study, a microdevice was developed to analyze the effects of culture medium composition in cell morphology and proliferation through imaging techniques [172]. Additionally, the utilization of patient-derived cells versus cell lines still needs to be further explored [173]. In the case of OS, since there is a high heterogeneity between patients, the cell source is an important aspect to consider. Cancer cell lines are a staple in cancer research, representing an almost unlimited source in comparison to the limited availability of patient-derived material. However, since multiple cell line-dependent results have been reported, selecting a proper cell line should be carefully analyzed for each study [174]. In contrast, patient-derived cells represent patient-specific features and have been extensively explored in the field of personalized medicine. Drawbacks related to their use include difficult culture conditions, short-term expansion, rapid loss of specific phenotypes in vitro, and high variability between donors [175]. Moreover, patient-derived cells are also relevant to introduce crucial TME components in novel 3D in vitro models. Tumor organoid protocols have greatly evolved to tackle some of these problems, and some studies, including for OS, have reported the inclusion of immune cells, allowing immunotherapy testing [144, 176]. Finally, improving these technologies' reproducibility and high-throughput capability is another obstacle to overcome, which includes avoiding batch-to-batch variations of

animal-sourced components, such as fetal bovine serum or Matrigel® [177, 178]. Human derivatives are already being explored, including the utilization of xeno-free alternatives for cell culture from human origin [177], synthetic biomaterials, or human-derived composite materials, such as PLMA hydrogels, which have proven successful for OS drug screening [102, 103]. In addition, the emerging applicability of 3D bioprinting in the design of complex and reproducible 3D constructs has been a crucial breakthrough in tissue engineering and cancer research. For OS, this technology has not yet been employed; nonetheless, its advantages, including the precise spatiotemporal deposition of cells and biomaterials under a well-defined architecture, its microscale resolution, and commercial availability, make it a promising technology to be explored [179–181]. Moreover, 3D bioprinting has been used to aid the fabrication of MCTS in a high-throughput fashion, which also highlights the ability to culture multiple cell types (such as fibroblasts, cancer, and endothelial cells) within specific niches [182]. For bone-related studies, experiments performed with OS cell lines have shown the possibility to incorporate OS cells into printable bioinks composed of alginate, gelatin, polyphosphate, and agarose or biosilica [183, 184]. For metastasis modeling, stereolithography-based 3D printing has been applied to create a breast cancer model consisting of an osteoblast-laden nanostructured bone matrix, a vascular channel, and

a tumor region [185]. The bioinks used (GelMA, PEGDA, HA nanocrystals) were optimized for cell survival and the cancer cells were able to migrate through the endothelial channel into the bone matrix.

Despite the benefits of 3D-based assays, the transition from 2D cultures to novel culture systems has been rather slow. Some reasons that can justify this slow transition include the higher cost of these technologies, the difficulty to adapt to HTS, complex optimization steps associated with low reproducibility, the powerful imaging technology necessary, and limited accessibility in some research laboratories. In addition, given the wide range of available culture platforms, it is challenging to compare results between different 3D and 2D cultures, and there is a clear lack of comparison with patient responses, which is essential for translating and validating these novel models. Besides, the complexity of these models will definitely grow with the addition of multiple cell types, ECM components, and biomaterials, which will further hinder downstream analysis since, for instance, some cells do not express specific markers. Therefore, it is necessary to find a balance between complexity and readability to develop physiologically relevant models that can be easily analyzable. Fortunately, some of these hurdles are already being undertaken and there are now a growing number of commercial products related to 3D cell culture and even metastasis modeling in vitro, such as ECM-mimicking hydrogels, spheroid-related products, and microfluidic platforms [186, 187], which may contribute to a wider acceptance and exploration of these platforms' potential in more research laboratories.

Overall, preclinical research still relies mostly on 2D and animal models, especially for anti-cancer drug screening. The complexity and physiological relevance of animal models are still unmatched in vitro. Therefore, reliable animal models are still needed to evaluate the metabolism and efficacy of novel drugs, to study the metastatic cascade of cancer as well as to validate novel 3D in vitro platforms. Besides, in vivo studies coupled with novel technologies such as intravital video microscopy and ex vivo approaches (tumor organoids, PuMA assay) can enhance the refinement of animal experiments related to cancer metastasis. In addition, bioengineered 3D models are also appropriate assays to reduce unnecessary in vivo studies. By allowing a better representation of the tumor's pathophysiology in vitro, these novel 3D models can be used at an early preclinical stage to eliminate possible drug candidates and to further improve our knowledge of cancer-related events, including the metastatic spread of tumor cells or the resistance to therapeutics. In the specific case of OS, most of the presented technologies remain unexplored. As most of the OS deaths are related to pulmonary metastases and given the relevant role of TME in metastasis formation, many of these platforms can be adapted to properly model the OS TME in vitro and

better understand the events of OS origin, progression, and metastasis as well as to find novel targets to develop more effective treatments in the future.

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Declarations

Competing interests The authors declare no competing interests.

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