

Strategies for Constructing Osteosarcoma 3D Culture Models and Progress in Their Applications

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Abstract: Osteosarcoma is a highly malignant primary bone tumor, and traditional two-dimensional (2D) culture methods cannot accurately simulate the tumor microenvironment. Three-dimensional (3D) culture better simulates the in vivo tumor microenvironment, providing more realistic experimental data regarding tumor progression, drug resistance, and drug screening. This paper introduces various 3D culture technologies, including scaffold-free culture, scaffold-based culture, microfluidic chips, and bioprinting. Moreover, 3D models show significant advantages in tumor microenvironment simulation, drug screening, and research on osteoblasts and osteoclasts, thus promoting the development of personalized therapy. Overall, 3D culture offers an important tool and platform for a deeper understanding of osteosarcoma's biological mechanisms and improving clinical treatment outcomes.

Keywords: Osteosarcoma; Three-dimensional Culture; Spheroid Culture; Scaffold Matrix; Bioprinting; Microfluidic Chips; Drug Screening; Personalized Therapy.

1. Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor, with an incidence rate of 0.8-0.9 cases per 100,000 people per year. Osteosarcoma mostly occurs in children and adolescents, with a slightly higher incidence rate in males than females. Its occurrence is associated with multiple factors, including genetic susceptibility and environmental influences. For example, previous radiotherapy (RT), Paget's disease, and hereditary conditions such as Li-Fraumeni syndrome can all increase the risk of osteosarcoma. Typical clinical manifestations of osteosarcoma include localized pain, swelling, and limited mobility, often involving long bones like the distal femur, proximal tibia, and proximal humerus [1][2]. Osteosarcoma is highly invasive and prone to early hematogenous metastasis, particularly to the lungs, which is the main reason for poor prognosis. Many patients have detectable metastatic lesions at the time of diagnosis, while the proportion of occult metastases is even higher. The standard treatment for osteosarcoma currently includes a combined treatment mode of neoadjuvant chemotherapy, surgical resection, and postoperative adjuvant chemotherapy [3]. However, this approach still has limitations in improving local control and prolonging survival. For patients without metastasis, the 5-year survival rate is 60%-70%, whereas for those with metastasis, the 5-year survival rate remains below 30% [4, 5]. Additionally, the toxicity of chemotherapy drugs can lead to severe side effects, negatively affecting quality of life [6, 7].

The mechanisms behind osteosarcoma progression, metastasis, invasion, and colonization are not yet fully understood, which is essential for developing therapeutic strategies. Previously, two-dimensional (2D) standard models (e.g., on plastic or glass surfaces) were widely used due to their simplicity, high reproducibility, and cost-effectiveness

[8]. However, tumor cells in the human body exist in a three-dimensional (3D) structure, constantly interacting with each other and with the extracellular matrix (ECM). The 2D culture cannot simulate the true growth environment of tumor cells in vivo [9, 10]. Moreover, the signaling cascades observed during tumor initiation and progression in 2D culture systems may be limited, potentially obscuring the mechanisms of drug resistance and leading to erroneous conclusions, thus complicating our understanding of cancer biology [11]. Many studies have reported that certain aspects of tumor cell biology cannot be explained using conventional 2D methods. During traditional drug screening based on 2D culture, many drugs deemed effective did not show corresponding efficacy in vivo, which might be attributed to the failure of 2D systems to replicate the physiological structure, mechanical properties, heterogeneity, and drug penetration of tumors [12, 13, 14].

In general, cells often lose their ability to differentiate autonomously after adhering to a surface, but osteosarcoma contains a unique population of cancer stem cells (CSCs) capable of forming suspended spheroid colonies under non-adherent and serum-deprived conditions [15]. This feature allows us to cultivate osteosarcoma cell lines in 3D. Compared to 2D culture, 3D culture preserves tumor cell heterogeneity and stem cell characteristics, providing a more physiologically relevant platform for new drug screening and personalized therapy. Osteosarcoma heterogeneity is believed to be a key factor in the development of drug resistance during treatment. In addition, cancer stem cells, the tumor microenvironment (TME), the extracellular matrix (ECM), and immune infiltrating cells all play crucial roles in osteosarcoma progression and drug resistance [16].

The tumor microenvironment is a complex dynamic ecosystem composed of various physical signals and biochemical factors that promote cancer progression, making osteosarcoma research particularly challenging. Animal

xenograft models have limitations due to ethical concerns, high costs, and cross-species differences that hinder clinical translation. Therefore, to better understand osteosarcoma progression, particularly in drug resistance studies, 3D culture platforms capable of replicating the natural characteristics of osteosarcoma have become an ideal choice [17].

This study aims to systematically evaluate the applications of different 3D culture methods in osteosarcoma research and to explore their advantages in simulating the tumor microenvironment, screening antitumor drugs, and understanding mechanisms of drug resistance. By comparing scaffold-free culture, scaffold-based culture, microfluidic chips, and bioprinting techniques, this paper attempts to provide scientific support for selecting the most suitable 3D culture method and offers new insights into personalized therapeutic strategies for osteosarcoma.

2. Strategies for Constructing Osteosarcoma 3D Culture Models

The goal of the osteosarcoma 3D culture model is to simulate the actual tumor microenvironment, retain the characteristics of tumor cells, and provide stable conditions for cell proliferation and metabolism. According to different culture techniques, osteosarcoma 3D culture systems can be divided into four main types: scaffold-free culture, scaffold-based culture, microfluidic cell chips, and bioprinting.

2.1. Scaffold-Free Culture

Scaffold-free culture is an important approach for 3D culture, mainly involving liquid overlay techniques and hanging drop techniques to form tumor spheroids. These techniques do not require scaffold materials to support, relying instead on gravity or other physical factors to allow cells to spontaneously aggregate into spheroids. For example, the hanging drop method can form spherical tumor colonies in a short time, although long-term preservation of these pure cell aggregates is challenging. Additionally, scaffold-free culture is simple and suitable for high-throughput screening; however, due to the lack of scaffold structure, its physiological reproducibility is relatively poor, limiting its application.

For example, Jubelin et al. used the liquid overlay technique (LOT) and hanging drop (HD) to cultivate highly reproducible MNNG/HOS osteosarcoma 3D tumor spheroids on round-bottom and flat-bottom multi-well plates. They found that although the LOT method is simple and inexpensive, suitable for high-throughput screening, it is also an effective method for cultivating a 3D model better suited for drug response measurement [18]. Susumu et al. used PrimeSurface 96 U plates to culture 3D osteosarcoma spheroid models to study chemoresistance treatment targets [19]. Li et al. used a 96-well ultra-low attachment plate (ULA plate) to culture tumor spheroids to validate the effect of bisphosphonate-loaded micro-arc oxidation (MAO) coated degradable magnesium-strontium (Mg-Sr) alloy particles in treating highly malignant osteosarcoma [20]. Additionally, Christiane Gebhard et al. found that although the overall level of STIP1 protein did not significantly differ between monolayer and spheroid cultures of canine osteosarcoma, its distribution changed. STIP1 was expressed only in the cytoplasm in monolayer cells but was localized in the nucleus in tumor spheroids [21]. Ozturk et al. cultured SaOS-2 osteosarcoma in non-adhesive agarose gel and showed that

the stem cell phenotype was maintained longer in 3D culture, facilitating more relevant drug screening and improved targeted treatment efficacy [16].

The tumor spheroid model based on scaffold-free culture represents an in vitro model of micro-tumors and metastases at an avascular stage. Although scaffold-free culture is simple and easy to use, the lack of scaffold structure limits its application in simulating complex in vivo tumor microenvironments.

2.2. Scaffold-Based 3D Culture

Scaffold-based 3D culture uses natural or synthetic scaffolds to provide physical support for tumor cells. Natural scaffolds typically include ECM components such as collagen, laminin, and gelatin, while synthetic scaffolds include materials like polyethylene glycol (PEG) and poly(ϵ -caprolactone) (PCL). Scaffold-based culture helps provide a physical and biochemical environment similar to in vivo conditions, thus better simulating tumor growth and invasion processes. For instance, research has shown that osteosarcoma cells cultured with gelatin or Matrigel matrix exhibit higher gene expression and tumor-related phenotypes [13].

Massimini et al. used rat tail tendon type I collagen to prepare 3D cultures of the canine D17 osteosarcoma cell line to study vasculogenic mimicry (VM). They found that after 48 hours of culture, D17 cells mimicked endothelial cell growth, forming tubular networks characteristic of VM, and created hollow matrix channels in long-term 3D culture while expressing heat shock protein 90 (Hsp90) and vascular endothelial growth factor receptor 1 (VEGFR1), but lacking endothelial marker CD31 expression [22]. Tome et al. demonstrated through fluorescence imaging that osteosarcoma cells cultured in Gelfoam® (gelatin sponge) and Matrigel formed three-dimensional osteosarcoma tissue structures [23]. Matrigel, a classical 3D culture substrate rich in ECM proteins such as laminin, type IV collagen, and growth factors, mimics the physiological environment of osteosarcoma [13]. Research has shown that osteosarcoma cells cultured in Matrigel are morphologically more similar to their original tissues, reflecting the actual processes of osteosarcoma progression [24].

Furthermore, Nie et al. used fresh patient-derived osteosarcoma samples for organoid modeling in Matrigel and treated the organoids with or without GPC3 expression using immunotherapy. Results indicated that GPC3 might be an effective therapeutic target for osteosarcoma [25]. Letitia K et al. co-cultured human MG-63 osteosarcoma cells with macrophages in post-electrospun PCL scaffolds, which had adjustable stiffness, in a bone tumor niche. They found that tumor-associated macrophages conferred highly variable biochemical signals in the 3D model, suggesting that the immune system should be considered when developing and testing new cancer drugs [26]. He et al. developed honeycomb-like Gelatin Methacrylate (GelMA) hydrogel microspheres using synchronous photopolymerization microfluidic technology. Results showed that osteosarcoma cells (K7M2) cultured in 3D GelMA microspheres exhibited increased tumor stemness, proliferation, and migration ability, as well as chemotherapy resistance to doxorubicin (DOX), and higher tumorigenicity in immunocompetent mice [27].

Eric R et al. used poly(ϵ -caprolactone) (PCL) and gelatin coaxial electrospun fiber meshes as scaffolds to model the human MG63 osteosarcoma cell line in 3D. They developed

a 3D osteosarcoma model with tunable mechanical properties, demonstrating significant effects of matrix stiffness and tissue structure on osteosarcoma. For instance, as 3D stiffness decreased, nuclear YAP/TAZ increased, and a downregulation of the IGF-1R/mTOR cascade and upregulation of Sox2 was observed in 3D environments, suggesting increased resistance to IGF-1R/mTOR-targeted chemotherapy and enhanced stemness [28].

Scaffold-based 3D culture offers important advantages for drug screening and resistance research by providing physical support and replicating in vivo microenvironments, thereby enabling tumor cells to exhibit more in vivo characteristics.

2.3. Microfluidic Chips

Microfluidic technology precisely controls fluid flow and integrates multiple functions, providing a relatively consistent and stable platform for 3D culture, enabling the systematic study of tumor behavior and progression under perfusion conditions. 3D cell culture systems based on microfluidic devices have the advantages of high throughput, batch feeding, and controlled perfusion, which are more controllable in terms of nutrient supply, aeration, cell growth, and intervention, with minimal external interference, thus achieving statistically significant sample sizes [29]. For instance, Sarkar et al. used polydimethylsiloxane (PDMS) microfluidic devices to successfully culture and harvest tumor spheroids of uniform shape and size and used multiphoton laser scanning microscopy (MPLSM) to study the effects of endothelial cells and conditioned medium on oxygen tension within live tumor spheroids [30]. Fois et al. constructed a microfluidic culture platform using microwells with integrated vascular beds for vascularization studies of hMSC and MG-63 cell spheroids, effectively simulating the vascularization and nutrient supply processes in the tumor microenvironment [31].

2.4. Bioprinting Technology

Bioprinting technology (BioP) constructs artificial 3D cell microenvironments by spatially depositing bioinks. Bioinks consist of biological materials and cell components. It is essential to ensure biocompatibility in BioP by using matrices with appropriate stiffness and porosity to regulate cell behavior. Giada et al. successfully constructed 3D cultures of osteosarcoma cells using functionalized hydrogels composed of gelatin and chitosan, along with UMR-106 rat osteosarcoma cells, via bioprinting [32]. Lin et al. used GelMA mixed with osteosarcoma cells for 3D bioprinting to generate osteosarcoma-like tumors, and they found that, compared to 2D culture, the 3D model showed significant changes in pathways related to cell cycle, metabolism, adhesion junctions, and epigenetic regulation, and was more sensitive to therapies targeting autophagy pathways [33]. In another study, Lin et al. adjusted the stiffness of the GelMA matrix and found that osteosarcoma cells proliferated faster and migrated more actively in softer 3D matrices, while secreting more MMP and VEGF in stiffer matrices, potentially promoting vascular invasion and affecting the integrin α 5-MAPK signaling pathway [34]. Shahn-Shamsabadi et al. combined collagen and cells to form various shapes of 3D cell aggregates, including spheres, cubes, dumbbells, and crosses, using 3D printing technology with PDMS molds, and they were able to maintain these shapes during long-term culture. The study showed that this technique could precisely control the spatial position of

different cell types within the construct, achieving highly customized cell models [35].

Bioprinting technology provides more refined 3D models for osteosarcoma research, allowing researchers to create microenvironments that more closely mimic in vivo conditions by adjusting biomaterials and printing parameters. These models have great potential for simulating the complex biological behavior of tumors, studying the impact of the tumor microenvironment on cancer progression, and screening new drugs.

3. Applications of Osteosarcoma 3D Models

3.1. Tumor Microenvironment

The tumor microenvironment is a complex three-dimensional network composed of the extracellular matrix (ECM), vasculature, and immune cells in a dynamic ECM. The matrix carries multiple signals, including structural signals, cell interactions, biochemical signals, and mechanical cues. In addition to osteosarcoma cells, the tumor microenvironment consists of osteocytes, stromal cells, vascular cells, immune cells, and calcified ECM. This complex and dynamic microenvironment is one of the reasons osteosarcoma becomes resistant to traditional therapies [26]. When evaluating the tumor microenvironment, precise control and reproducibility are key factors, including temperature, pH, flow rate, nutrient and oxygen supply, metabolic waste removal, and mechanical loads on cells. These conditions directly affect tumor behavior, with the mechanical environment regulating the lineage differentiation of normal mesenchymal stem cells (MSCs) and the generation of cancer stem cells.

Currently, the high failure rate of drugs for osteosarcoma in early clinical trials can be partly attributed to the lack of effective tumor microenvironment simulation in existing in vitro models. Therefore, reconstructing the tumor's living environment is a primary task in in vitro research. Different in vitro culture techniques (including physical factors such as ECM stiffness and structure) significantly affect cell phenotypes, biomarker expression, and therapeutic resistance. Compared to traditional two-dimensional (2D) culture, three-dimensional (3D) culture models have a significant advantage in simulating the tumor microenvironment, providing a structure closer to the in vivo environment. 3D culture models have demonstrated better effectiveness in studying tumor cell invasion, metastasis, and resistance. Osteosarcoma shows more realistic proliferation, metabolism, angiogenesis, resistance, gene expression, and protein expression in 3D culture than in 2D culture [36]. For example, Giada Bassi et al. demonstrated that osteosarcoma stem cell marker genes such as OCT-4, NANOG, and SOX-2 were significantly upregulated in biomimetic conditions, suggesting that the 3D bioactive environment could effectively maintain the pluripotency and self-renewal capacity of osteosarcoma stem cells [37]. González Díaz et al. also found that 3D culture better retained the genetic phenotype of osteosarcoma compared to 2D culture [12].

3.2. Drug Screening and Resistance Research

3D culture has significant advantages in drug screening and resistance research. Many drugs screened using 2D systems do not exhibit the corresponding efficacy in vivo, whereas 3D culture systems can better replicate drug penetration and its

effects on tumor tissue, improving drug screening effectiveness. For instance, González Díaz et al. compared 2D, 3D (3D Gel and GelHA μ RB), and patient-derived xenograft (PDX) models and found that only 3D Gel and GelHA μ RB retained IGF-1R phosphorylation, associated with resistance, observed in PDX-derived cells. In contrast, 2D cultures lost the resistance signals. The 3D GelHA μ RB model could identify drug targets that traditional 2D cultures or 3D models lacking bone mineral cues might miss, highlighting a significant improvement over traditional osteosarcoma models for in vitro research [12]. Lin et al. conducted multi-omics analysis based on 3D bioprinted models and identified distinct osteosarcoma cell metabolic features and drug sensitivity, innovating the discovery of therapeutic targets for osteosarcoma [33]. Tumor engineering models could restore the hypoxic and glycolytic tumor phenotypes of osteosarcoma and re-express adaptive features of angiogenesis and vasculogenic mimicry [38]. The compaction of 3D spheroids contributes to increased half-maximal inhibitory concentration (IC₅₀) in 3D drug testing, while ECM serves as a physical barrier in tumors, limiting drug penetration [10]. Although mass spectrometry imaging (MSI) showed sufficient diffusion of chemotherapeutic drugs within 3D osteosarcoma structures, doxorubicin exhibited significantly lower cytotoxicity in 3D constructs compared to conventional 2D cultures [29]. This suggests that drug screening in 3D systems better reflects in vivo conditions than 2D culture, emphasizing the importance of using 3D models for drug screening [13].

3.3. Research on Osteoblasts and Osteoclasts

Osteosarcoma development is closely associated with osteogenesis and osteoclastogenesis. In 3D models, researchers can simulate the bone microenvironment using various scaffold materials to study the roles of osteoblasts and osteoclasts during tumor progression. Different types of osteogenic cells can be used in in vitro osteogenesis studies, including human osteosarcoma (HOS) cells, Saos-2 cells, mouse osteoblastic MC3T3-E1 cells, rat cranial osteoblasts, and rat mesenchymal stem cells (BMSC). When studying osteogenic differentiation of MG63 cells, standard cell culture plates are typically used, and special reagents such as ascorbic acid and dexamethasone are added. The standard protocol for primary BMSC osteogenesis also includes dexamethasone and ascorbic acid, along with bone morphogenetic protein (BMP)2 and β -glycerophosphate. Among these, dexamethasone mainly promotes cell proliferation, while ascorbic acid induces alkaline phosphatase (ALP) and osteocalcin expression.

During osteogenic differentiation, osteoblasts produce type I collagen, osteocalcin, osteopontin, and ALP. ALP is upregulated during the early stage of osteogenic differentiation, while osteocalcin is predominantly expressed during the mineralization stage. Belka et al. used Fe-MEPE (metal supramolecular coordination polyelectrolyte) films as substrates and successfully induced osteogenic differentiation of MG63 and BMSC without adding special reagents, offering a promising scaffold material for bone defect repair [39]. Kihara et al. found that HOS cells cultured on a 2D culture plate with β -glycerophosphate (β -GP) formed spherical mineralized matrices of about 12 μ m in diameter after 7 days of culture. In contrast, amorphous mineralized particles of about 1.7 μ m formed in just 3 days in 3D collagen gel with β -GP. These mineralized particles formed not only in

type I collagen gel but also in Panacea Gel (peptide synthesis hydrogel). The study further found that the turbidity and calcium content of the gel correlated positively, allowing estimation of the precipitation of mineralized particles by measuring gel turbidity. Although the mechanism of HOS cell mineralization in 2D and 3D cultures is the same (i.e., HOS cell mineralization is not merely a calcium phosphate precipitation reaction but involves cellular biological processes), the shape and formation time of the mineralized particles differed significantly. Furthermore, studies have shown that regardless of the type of hydrogel, osteogenic cells in 3D hydrogels can form mineralized particles. Therefore, 3D osteosarcoma culture can serve as an in vitro model for osteogenesis research, aiding in understanding the mechanisms of osteosarcoma invasion in bone tissue [40].

3.4. Personalized Therapy

3D culture has shown great potential in personalized therapy. Constructing personalized 3D models using patient-derived cells allows for more precise predictions of patient sensitivity to different treatment regimens [12]. For example, honeycomb-like GelMA microspheres, due to their high efficiency and biocompatibility, can be used to establish personalized tumor models that precisely simulate the patient's tumor microenvironment. This characteristic makes it possible to rapidly screen multiple chemotherapy and targeted therapy options, making personalized treatment more targeted and effective [27].

Due to the high heterogeneity of osteosarcoma, personalized therapy is crucial for improving patient survival rates. 3D models established from patient-derived tumor cells or biopsy samples can simulate the specific characteristics of the patient's tumor, aiding in predicting the effectiveness of particular treatment options. For instance, patient-derived tumor organoid (PDO) models enable researchers to evaluate the efficacy of different drug combinations, thereby helping formulate personalized treatment plans for patients. Patient-derived 3D tumor models can bridge the gap between preclinical and clinical studies, advancing osteosarcoma research and personalized therapy [41].

4. Conclusion and Future Perspectives

The 3D culture models of osteosarcoma provide an important tool for understanding its biological mechanisms and improving treatment strategies. Compared to traditional 2D culture systems, 3D systems have significant advantages in simulating the tumor microenvironment, drug screening, and resistance research. Despite challenges such as material selection and operational complexity, their potential applications in biological research and personalized therapy are enormous.

3D osteosarcoma models can simulate the in vivo tumor microenvironment, chemotherapy resistance, and differential signaling expressions. Compared to 2D systems, 3D models exhibit key differences in biological behavior, with drug responses closer to in vivo conditions and gene expression patterns being more representative. Specifically, in a 3D environment, increased cell proliferation rates require tumor cells to cope with stress conditions like hypoxia and nutrient deprivation, which makes 3D culture resemble the actual conditions of aggressive tumors more closely. Additionally, the tissue topology and cell interactions in 3D systems are more biomimetic, and the bone niche plays a vital role in preserving signal transduction and drug response in

osteosarcoma. 3D culture helps us better understand the progression of osteosarcoma, clarify cellular molecular mechanisms, and improve clinical translation efficiency. The 3D culture technology also has the potential for precise control of environmental conditions and high reproducibility, such as controlling temperature, pH, flow rate, nutrient and oxygen supply, metabolic waste removal, and mechanical load. This helps simulate a real tumor niche, revealing mechanisms of gene and protein expression, tumor growth, invasion, metastasis, and exploring potential therapeutic strategies, which is the ultimate goal of establishing in vitro 3D osteosarcoma models.

Although 3D osteosarcoma models have limitations and cannot fully reproduce the actual tumor niche, these simplified models provide opportunities to explore the cellular and molecular interactions of osteosarcoma in a more realistic context. Future research should further optimize the construction of 3D culture models, especially in terms of scaffold material selection and precise control of the cellular microenvironment, to improve their physiological relevance and reproducibility. Additionally, combining 3D culture with high-throughput drug screening, genomics, and metabolomics will help uncover the underlying mechanisms of osteosarcoma and accelerate the development of new therapeutic drugs. Through continuous technological innovation and multidisciplinary collaboration, 3D culture technology will play an increasingly important role in the study of osteosarcoma and other tumors, bringing more treatment options and hope to patients.

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