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Review

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From 2D to 3D: transforming malignant bone tumor research with advanced culture models

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Abstract: Osteosarcoma (OS), chondrosarcoma (CS), and Ewing sarcoma (ES) represent the primary malignant bone tumors and pose significant challenges in oncology research and clinical management. Conventional research methods, such as 2D cultured tumor cells and animal models, have limitations in recapitulating the complex tumor microenvironment (TME) and often fail to translate into effective clinical treatments. The advancement of 3D culture technology has revolutionized the field by enabling the development of in vitro constructed bone tumor models that closely mimic the in vivo TME. These models provide a powerful tool for investigating tumor biology, assessing therapeutic responses, and advancing personalized medicine. This comprehensive review summarizes the recent advancements in research on *in vitro* constructed 3D tumor models for OS, CS, and ES. We discuss the various techniques employed in model construction, their applications, and the challenges and future directions in this field. The integration of advanced technologies and the incorporation of additional cell types hold promise for the development of more sophisticated and physiologically relevant models. As research in this field continues to evolve, we anticipate that these models will play an increasingly crucial role in unraveling the complexities of malignant bone tumors and accelerating the development of novel therapeutic strategies.

Key words: 3D culture; Disease model; Osteosarcoma; Chondrosarcoma; Ewing sarcoma

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1 Introduction

Bone tumors, emanating from diverse cellular constituents intrinsic to the osseous tissue or manifesting within its confines, represent a category of intricate and challenging maladies within the clinical domain(Huang et al., 2023; Ma et al., 2023). The distinctive biological and clinical characteristics of bone tumors make them a focal point of investigation in medical research. The spectrum of bone tumor subtypes notably includes osteosarcoma (OS), chondrosarcoma (CS), and Ewing sarcoma (ES) (Anderson and Doyle, 2021). Patients typically manifest with symptoms encompassing pain, pathologic fractures, localized masses, and functional impairment(Jimenez-Andrade et al., 2010). The prognosis is frequently unfavorable, posing a formidable challenge to the improvement of clinical treatment protocols.

Current therapeutic approaches for bone tumors rely on a multidisciplinary and integrated treatment modality, including surgical intervention, radiotherapy, and chemotherapy(Shao et al., 2022). Nevertheless, conventional therapeutic modalities exhibit evident constraints when confronted with the profound

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heterogeneity and aggressive nature of bone tumors. Collectively, surgical trauma, chemotherapy-associated toxicities, and radiotherapy-induced damage to normal tissues have a substantial adverse impact on patients' quality of life and treatment efficacy(Shao, et al., 2022). Although many preclinical tumor models (e.g., patient-derived xenograft models) are currently available for corresponding drug development, research on rare tumors (e.g., malignant bone tumors) is often limited by the high costs involved (Xue et al., 2020). There is, therefore, a pressing need to enhance our understanding of bone tumors and develop more precise and cost-effective interventions.

The maintenance of bone strength depends on the presence of type I collagen and mineral crystals within the bone matrix, which contains pivotal growth factors such as insulin-like growth factor (IGF), bone morphogenetic protein (BMP), transforming growth factor β 1 (TGFβ1), and platelet-derived growth factor (PDGF)(Hauschka et al., 1986; Florencio-Silva et al., 2015; Eguchi et al., 2018; Verrecchia and Redini, 2018). The sophisticated regulatory network is commonly perturbed during bone tumorigenesis, giving rise to anomalous proliferation of osteocytes and pathological alterations in bone tissue. This disruption occurs at various levels, including genetic alteration, aberrant cell signaling, and maladaptation within the microenvironment. The tumor microenvironment (TME), a complex and dynamically evolving system comprising cellular components and noncellular components, assumes a significant role in tumor progression and the efficacy of drug therapy, constituting a dynamically evolving and intricate system(Xiao and Yu, 2021; Zubair et al., 2022).

Two-dimensional (2D) cultures and animal models are now widely used by the scientific community in oncology research. In recent years, driven by rapid advancements in biological tissue engineering and cellular biotechnology, the technology of 3D culture tumor models has emerged. This novel technology allows for a more precise examination of tumor growth mechanisms and developmental processes, offering a more authentic and complex tool for investigating bone tumors(Habanjar et al., 2021; Wood and Ewald, 2021). Each of these three methods has its own advantages and disadvantages, which we briefly summarize in **Table 1**.

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Table 1 Comparison among 2D and 3D culture and animal model

Methods	Advantages	Disadvantages
2D culture	● Facile operational procedures ● Low cost ● High-throughput	● Lack of cell-cell and cell-matrix interactions ● Cannot simulate tissue structure and microenvironment(Marques et al., 2022).
Animal model	● High biological similarity ● Assess overall biological responses	● High cost ● Time-consuming ● Ethical issues(Lui et al., 2011) ● Intrinsic immunological disparities(Kelland, 2004)
3D culture	● Closer to *in vivo* conditions ● Providing more accurate drug screening results ● Cost-effectiveness ● Abundant cell-cell and cell-matrix interactions(Maltman and Przyborski, 2010)	● Difficult to scale up production ● Requires special equipment and technical support

Three-dimensional (3D) culture tumor models are widely applied in various fields, with culture methodologies such as organoids(Sato et al., 2009), spheroids(Costa et al., 2014), and tumor-on-chip(Wan et al.,

2020) offering a novel perspective for investigating bone tumors. Despite their potential as precise models for therapy response, the advancement of 3D tumor organoids is hindered by technical constraints and the extensive manipulations required by current methods. Patient-derived xenograft models aim to better recapitulate human cancers yet are still constrained by the large cost and time associated with their use, which translates to the impracticality of performing large drug screenings. High-speed live cell interferometry (HSLCI) has been developed with the organoid screening approach, which combines automated cell seeding via bioprinting for non-invasive, label-free, time-resolved imaging(Phan et al., 2019; Tebon et al., 2023). Concerning the personalized advancement of bone tumor treatment, this technology furnishes a novel platform for drug screening and individualized therapeutic interventions(Nayak et al., 2023). Despite the significant potential of 3D-cultured tumor models in bone tumor research, they have inherent limitations. One challenge is the simulation of the authentic vascularization processes, which is essential for sustaining tumor growth. Another challenge is the co-culturing of multiple cells, particularly the complex interactions among diverse cell types in bone tissue, which requires further in-depth investigation (Habanjar, et al., 2021). Furthermore, although 3D models can recreate the three-dimensional structure of tumors, current models do not fully replicate the intricate *in vivo* growth environment due to its complexity, resulting in some deviation from the tumor growth process in the human body.

In this review, we thoroughly discuss the evolution of 3D-cultured tumor models and their profound implications for the realm of bone tumor research **(Fig. 1**). We conduct a thorough investigation into the recent advances and limitations of 3D culture technologies for OS, CS, and ES using examples such as organoids, spheroids, and tumor-on-chip models. Our objective is to highlight the groundbreaking achievements of 3D-cultured tumor models and their potential to advance bone tumor research. Finally, we discuss the existing challenge and future perspectives of establishing 3D-cultured tumor models for bone tumor research and the evolving landscape of clinical oncology. By addressing these aspects, we aim to provide a comprehensive understanding of the impact of 3D-cultured tumor models on bone tumor research and their potential to revolutionize the field of clinical oncology.

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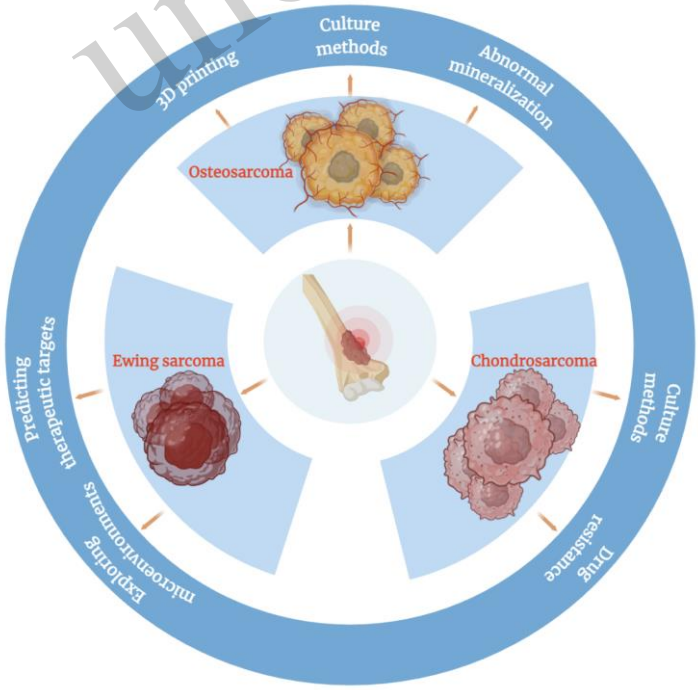

Fig. 1 Schematic overview showing what is discussed in this review about 3D models of malignant bone tumors represented by osteosarcoma, chondrosarcoma, and Ewing sarcoma in terms of disease research. Created in BioRender.com.

2 Osteosarcoma

Osteosarcoma (OS), a rare yet devastating solid tumor, possesses the capacity to manifest in any osseous structure, although it typically arises in the long bones, such as the distal femur, proximal tibia, and fibula. This malignancy primarily afflicts children and young adults, exhibiting a dismal 5-year survival rate that has remained stagnant over the past four decades. Upon metastasis, OS can disseminate either locoregionally or distantly to organs such as the lungs. The intricate architecture and surrounding TME pose significant challenges, as conventional 2D monolayer cultures fail to faithfully recapitulate the complexities of the natural tumor milieu, thereby impeding progress in OS research. However, the emergence of 3D culture technologies has provided a platform for advancing our understanding of the pathogenesis and treatment of OS(Aran et al., 2021; Wu et al., 2021; Cillo et al., 2022).

2.1 Three-dimensional culture methods for modeling OS

Recent advancements in tissue engineering have aimed to narrow the disparity between preclinical *in vitro* screening and *in vivo* models by developing sophisticated 3D *in vitro* bone cancer models, which offer a more physiologically relevant platform for evaluating therapeutic efficacy(Kureshi et al., 2015; Monteiro et al., 2020b). In the realm of 3D cultures, two primary categories have emerged: scaffold-free and scaffold-based approaches. Among the scaffold-free techniques, the hanging-drop method and liquid-based low-attachment cell cultures have garnered significant attention due to their ability to generate multicellular spheroids that closely mimic the 3D architecture and cell–cell interactions found within the TME. These methods have been extensively employed to study various aspects of OS biology, including drug screening, invasion, and metastasis. On the other hand, scaffold-based approaches utilize a variety of materials to support cell growth and provide a more physiologically relevant microenvironment. These scaffolds can be derived from natural or synthetic sources and can be engineered to mimic the extracellular matrix (ECM) found in the TME(Li et al., 2023). To systematically compare the advantages and disadvantages of the different methods for constructing *in vitro* models of bone tumors, we have summarized the key findings from previous studies in **Table 2** (Nath and Devi, 2016; Ferreira et al., 2018). This comparative analysis provides valuable insights into the strengths and limitations of each approach, enabling researchers to select the most appropriate method for their specific research questions.

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Table 2 Comparison of different methods for 3D malignant bone tumor modeling

	Advantages	Disadvantages
Hanging drop method	● Simple operation ● Easy model construction ● Reduced shear stress ● Good shape control ● Compatibility with standard liquid handling robots	● Difficulty in constructing large-size models ● Frequent medium changes ● Short incubation period
Ultra-Low Attachment method	● Long-term culture capability ● The high yield of spheroplasts ● Ability to multiplex with imaging	● Poor size/shape homogeneity ● Needs plate-coating procedure
Scaffold-based method	● Provide structural support ● Precisely regulate the form and dimensions ● Provide a suitable ECM	● Potential immunogenic or cytotoxic effects ● Interference with cell-cell interactions ● Asynchronous degradation with new tissue formation

3D printing	● Can be simultaneously used with imaging and other biochemical assays for multiplexing. ● Microfluidic device compatibility. ● Easily scalable ● Provide spatial control capabilities for biomaterials and cell components.	● Bioinks require optimization. ● Cell activity could be impaired ● Elevated cost associated with the device

2.1.1 Scaffold-free cultures for OS

Rimann and Laternser et al.established 3D microtissues consisting of OS cell lines SaOS-2, HOS, and MG-63 for anticancer drug screening using the hanging-drop method. In contrast to the corresponding cells cultured in 2D monolayers, the 3D cultures showed significantly higher IC50 values for doxorubicin, cisplatin, taurine, pemetrexed, and paclitaxel, suggesting that 3D microtissues more accurately reflect the sensitivity of OS to drug treatment(Rimann et al., 2014). Despite the widespread use of the hanging drop technique for rapidly forming tumor spheroids, it still falls short in replicating the natural TME that regulates cancer cell behavior. Moreover, the hanging drop method has several drawbacks, including inconvenient medium replacement and the potential for cell damage during manipulation. To address these limitations, Liu and colleagues developed a novel micro-hole culture chip (SimpleDrop) technique for creating 3D cell spheroids using the synovial sarcoma cell line HS-SY-II. This approach yielded results comparable to those of the traditional hanging drop method in drug screening but offered greater ease of manipulation and improved stability of the spheroids(Liu et al., 2021).

In addition to the hanging drop method, the Ultra-Low Attachment (ULA) approach has been employed to generate scaffold-free 3D OS models. Folry et al. constructed a model of OS using human OS cell lines and patient-derived human OS cells cultured in ULA plates. Interestingly, the 3D spheroids exhibited rapid development of chemoresistance and an altered growth rate compared to their 2D counterparts, suggesting that targeting epigenetic regulation in the early stages of latent metastasis could be a viable therapeutic approach(Foley et al., 2015).

2.1.2 Scaffold-based cultures for OS

Scaffolds play a dual role by providing structural support for tumor cell growth and replicating the ECM and TME. A wide array of synthetic materials like nano-hydroxyapatite (nHA), polyethylene glycol (PEG), polylactic acid-glycolic acid copolymer (PLGA), and polydimethylsiloxane (PDMS), as well as natural components, including collagen, matrix gum, silk, and agarose, are utilized either individually or in combination (polycombination) to create various scaffold types, such as sponges and hydrogels(Peela et al., 2017). Hydrogels, characterized by their softness (1-150 kPa), are extensively employed for 3D cancer modeling due to their ability to mimic a diverse range of biochemical and biophysical properties, enabling the visualization of 3D-cultured cells. Additionally, rigid biomaterials (MPa range), such as polyesters and ceramics, can be used to construct 3D bone tumor models. Several tissue engineering systems initially designed for promoting bone tissue regeneration have shown promise for mimicking bone cancer ecological niches *in vitro*, highlighting the traditional belief that a rigid matrix is essential for bone tissue formation(Pradhan et al., 2017).

Hydrogels, leveraging their ability to closely mimic the ECM, offer a three-dimensional aqueous microenvironment conducive to cell adhesion, proliferation, and enhanced cellular differentiation, rendering them indispensable in tissue engineering for 3D cultures. Furthermore, hydrogels can be customized by incorporating functional groups to tailor their properties accordingly. It is foreseeable that the biomimetic aqueous microenvironment facilitated by hydrogels will progressively enhance overall regulation, significantly streamlining the

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in vitro cultivation of bone tumors(Liu et al., 2022; Zhao et al., 2022; Wu et al., 2023). Recent studies have documented the use of methacryloyl platelet lysate (PLMA)-based hydrogels to sustain a humanized 3D OS spheroid model. This model exhibited enhanced drug resistance to doxorubicin compared to scaffold-free spheroids, highlighting the potential of PLMA hydrogels to facilitate tumor-invasive behavior and interactions with the TME. These findings underscore the potential of the integrated PLMA hydrogel system for complex tumor modeling(Monteiro et al., 2020a; Monteiro et al., 2021). Nevertheless, these hydrogels necessitate further modifications involving cell-adhesive components and degradable sites, and the preparation and purification procedures are time-consuming and technologically demanding. Hence, a straightforward and efficient alternative is the combination of natural proteins with synthetic polymers or polysaccharides.

Tornín et al. developed a 3D tissue engineering model of OS using scaffolds made of type I collagen and nHA. The model protects cells from oxidative stress damage by reducing the levels of reactive oxygen species and nitrogen in the TME and promotes epigenetic features in genes associated with the stemness of OS cells, demonstrating the response of the 3D culture model to plasma-activated fluid therapy(Tornín et al., 2021).

Gellan gum (GG)-silk filipin-blended spongy-hydrogels are emerging as a cost-effective alternative platform for 3D cancer modeling. OS spheroids, specifically Saos-2 cells, have demonstrated the ability to sustain metabolically active structures over prolonged periods within a 3D microenvironment formed by GG-silk fibroin hydrogels.

These spheroids exhibited heightened expression of key OS biomarkers, including osteopontin, osteocalcin, RUNX 2, and bone sialoprotein genes, along with robust alkaline phosphatase expression detected through immunohistochemistry, indicating their potential as a bionic ecological niche model for OS(Kundu et al., 2019). Collagen type I, matrigel, alginate, and agarose are commonly used scaffold materials that offer varying degrees of matrix stiffness and adhesion ligands. Jiang et al. explored the impact of matrix elasticity and adhesion on OS MG63 cells using hydrogels derived from these four materials. Their study revealed that 3D-cultured OS cells exhibited increased cell growth and tumor malignancy in stiffer hydrogels, such as collagen and agarose, compared to softer alternatives like matrigel and alginate(Jiang et al., 2020a). This finding emphasizes the significant influence of matrix stiffness and elastic modulus of the culture system on the biological characteristics of the tumor model. The strong dependence of OS cells on matrix stiffness is closely associated with the regulation of the integrin-mediated adhesion (FA) pathway.(Jiang et al., 2020b) Chitosan, a natural compound produced by deacetylation of chitin, has shown promise in drug delivery applications. A hydrogel based on chitosan and dipotassium orthophosphate has been reported to be effective in reducing the cytotoxicity of DOX in mice, particularly in terms of dermal and cardiotoxicity. This finding provides valuable insights for the development of 3D drug delivery models for OS(Maleki Dana et al., 2021).

Metastatic bone tumors are a critical area of clinical research, and the choice of 3D *in vitro* platform and its specific requirements depend on the stage of the metastatic cascade being studied, as the development of bone metastases is a complex, multi-step process. Recent studies have demonstrated that the ECM of metastatic bone tumors can be mimicked using hyaluronan-methacrylate (HA-MA) and GelMA photocrosslinkable 3D spheroid microgels fabricated on superhydrophobic surfaces. In experiments assessing cisplatin cytotoxicity, this 3D microgel-constructed microphysiological system exhibited greater resistance to platinum chemotherapeutic agents compared to mono-cultured or co-cultured 3D multicellular spheroids counterparts. This resistance profile more closely resembles that of metastatic bone tumors *in viv*o(Antunes et al., 2019). The demand for robust *in vitro* models that can effectively screen novel treatments for metastatic bone tumors is increasingly urgent. Suurmond et al. engineered a bone metastatic spheroid using methylcellulose and collagen type I, achieving rapid formation within 24 hours. This model was employed to evaluate the efficacy and specificity of cisplatin in treating metastatic bone tumors. Their results demonstrated that only cisplatin concentrations of 50 and 100 μM successfully inhibited the growth of these spheroids, with these concentrations aligning closely with therapeutic doses used in clinical settings. Advancements in 3D tumor models provide more information for enhancing the precision of in vitro therapeutic trial stage screenings, offering critical insights that inform the design of *in vivo* animal studies and subsequent clinical trial phases. This approach not only augments the

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probability of clinical trial approval but also bolsters the overall success rate of therapeutic interventions(Antunes, et al., 2019). Therefore, the development of robust, physiologically relevant micro-physiological systems that capture the complexity of the metastatic bone TME is crucial for the preclinical evaluation of novel therapies aimed at eradicating metastatic bone tumors.

2.1.3 Three-dimensional printing for OS

Three-dimensional printing technology is widely used as an innovative tool to manufacture tissue constructs in tissue engineering applications based on 3D CAD models. This technology enables precise control over the object's microstructure and offers design flexibility, thereby enhancing reproducibility(Roseti et al., 2017). Nonetheless, during the 3D printing process for constructing *in vitro* culture models, elevated mechanical forces may cause varying degrees of cellular damage, ultimately reducing cell viability. Consequently, a major challenge in bioprinting technology is the ability to print both cells and materials (bioinks) while maintaining cell viability. Collagen-based hydrogels and printing parameters that emulate the ECM of bone have been shown to maintain cellular metabolic activity.

Pellegrini et al. employed a 3D bioprinting platform to fabricate collagen hydrogels containing OS cells, which exhibited excellent biocompatibility. Subsequent experiments revealed that a U-2OS cell line model, developed using the specified collagen bioinks, demonstrated enhanced repair of drug-induced DNA damage and altered gene expression related to cisplatin chemosensitivity tests(Pellegrini et al., 2022).

Lu et al. used extracellular vesicles from human bone marrow-derived stem cells (hBMSC-EVs) and OS cells as bioinks to construct a micro-OS using 3D printing. In this model, the expression of tumor-specific markers (e.g., CD133 and MMP9) and bone-associated genes (e.g., OPN, ALP, and COL-1a1) was significantly elevated compared to the 2D culture approach, suggesting that the system simulated marrow niches to support the high aggressiveness and metastasis of OS cells(Lu et al., 2024).

A hydrogel consisting of biomimetic synthesized HAp nanocrystals and natural biomacromolecules (chitosan, L-arginine) was used by Zafeiris et al. as an ink during the printing process. This facilitated the attachment of MG63 human OS cells to the scaffold surface, leading to an increased cell count and high survival rate(Peela, et al., 2017). Bioscaffolds derived from collagen fibers exhibited low pore size or porosity. MG-63 human OS cells were cultured on bioscaffolds produced using 3D printing ink comprising a blend of hybrid collagen (Coll), nanofibrillated cellulose (NFC), carboxymethylcellulose (CMC), and citric acid (CA)(Dobaj Štiglic et al., 2023). These hybrid Coll bioscaffolds demonstrate excellent biocompatibility, stability, and structural support, rendering them a valuable asset in OS tissue engineering.

2.2 Three-dimensional OS model for probing abnormal mineralization of bones

While bone is known for its high mineral content, the specific role of bone minerals in OS progression and drug response remains elusive. Abnormal mineralized tissue formation is a hallmark of OS, with mitochondria serving as essential calcium carriers during osteogenesis. Rossi et al. found a change in mitochondrial morphology from elongated to rounded during differentiation of the SaOS-2 cell line, indicating potential metabolic reprogramming in OS cells that could lead to an enhanced dependence on glycolysis for energy metabolism(Rossi et al., 2023). Employing a 3D collagen scaffold, Picone et al. found, for the first time, that intracellular magnesium doping during the early stages of biomineralization positively influences the formation of hydroxyapatite (HA) platelets and the mineralization between fibrils(Picone et al., 2020). Emerging evidence indicates that the matrix rigidity of the culture system, with an elastic modulus ranging from 10^5 to 10^6 kPa in the mineralized bone microenvironment, modulates the gene expression of tumor cells to varying degrees(Vanderburgh et al., 2018). This modulation subsequently influences behaviors such as the growth and invasion of bone tumors, as well as the sensitivity to chemotherapeutic agents. Eva C González Díaz's team developed an OS model incorporating a bone-mimicking component to investigate the impact of 3D cultures and HA on OS signaling pathways and drug reactions. Their results affirm the significance of integrating bone mineral signals into OS experimental models to faithfully replicate the response of bone tumors to chemotherapy. This seminal work highlights the immense potential of 3D OS cultures as a novel approach for studying bone mineralization and unraveling the complex interplay between bone minerals and OS progression(González Díaz et al., 2022).

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2.3 Three-dimensional OS model to explore the immune microenvironment of bone tumors

The immune microenvironment plays a crucial role in the pathogenesis of bone tumors. TME-infiltrating monocyte-derived cells can be classified into three principal subsets: tumor-associated macrophages (TAMs), tumor-associated dendritic cells (TADCs), and myeloid-derived suppressor cells (MDSCs)(Ugel et al., 2021). TAMs, originating from circulating monocytes, are the most abundant immune cell population within the TME. They can be broadly categorized into two phenotypes: M1 and M2 macrophages. M1-polarized macrophages are traditionally associated with anti-tumor responses, primarily due to their secretion of pro-inflammatory cytokines. In contrast, M2 macrophages facilitate tumor progression by promoting angiogenesis and stromal remodeling, thereby enhancing tumor invasion and metastasis(Janes et al., 2021; Ugel, et al., 2021).

Bénédicte Brulin et al. assessed the proportions of immune and stromal cells within their organotypic models of mouse and canine OS. They revealed that these proportions closely resembled those observed *in vivo*. Furthermore, their study demonstrated that the 3D cell model exhibited a restricted therapeutic response to chemotherapeutic agents(Brulin et al., 2021).

Immunosuppressive cell-mediated immune escape is a primary factor contributing to the unfavorable prognosis of bone tumors. Myeloid-derived suppressor cells, regulatory T cells, and tumor-associated macrophages exert multifaceted regulatory effects on tumorigenesis and growth within the TME(Cersosimo et al., 2020; Huang et al., 2021). These interactions profoundly impact the proliferation, migration, invasion, and epithelial-mesenchymal transition of bone cancer cells and represent major targets for immunotherapeutic interventions. For instance, Marina et al. employed the hanging-drop method to generate three-dimensional spheroids of OS co-cultured with M2 macrophages and examined the migratory and invasive characteristics of this model. This 3D OS model contributes to a deeper understanding of the immune microenvironmental role of OS and the mechanisms of OS interactions with immune cells and biological matrices, advancing personalized medicine(Pierrevelcin et al., 2022).

In a similar vein, the induction of an inflammatory milieu by macrophages, characterized by high levels of tumor necrosis factor α (TNFα) and interleukin (IL)-6 within moderately stiff scaffolds, led to increased expression of Yes-associated protein (YAP) in co-cultured OS cells. However, this alteration did not significantly impact the cells' resistance to chemotherapeutic agents(Pierrevelcin, et al., 2022). These findings suggest that the signals conferred by TAMs in OS, which contribute to the inflammatory environment, exhibit considerable variability. Given this variability, it is crucial to consider both the biochemical cues within OS and signals from the immune system when developing and evaluating new cancer therapies, as well as when constructing 3D models of bone tumors.

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3 Chondrosarcoma (CS)

Chondrosarcoma (CS), a malignant tumor arising from cartilage tissue, is the second most prevalent malignant bone tumor, displaying an age-related incidence that peaks in the elderly population. A defining characteristic of CS is its high resistance to both chemotherapy and radiotherapy, leaving surgery as the sole effective treatment option. CS cells exhibit the unique ability to generate cartilage and possess a strong propensity for differentiation into hyaline cartilage, often accompanied by mucoid transformation, calcification, and ossification. The presence of membrane-bound P-glycoprotein has been correlated with more aggressive and higher-grade CS(Simard et al., 2017), while mutations in the isocitrate dehydrogenase genes IDH1 and IDH2 are frequently observed in CS. Despite these findings, the precise mechanisms governing CS differentiation remain unknown. To address this critical knowledge gap, numerous researchers have focused their efforts on investigating CS using advanced 3D culture models, which more accurately recapitulate the complex TME compared to traditional 2D cultures(Van Oosterwijk et al., 2013; Whelan and Davis, 2017).

3.1 Three-dimensional culture method for CS

The hypoxic physiological microenvironment has made sphere culture the primary method for the 3D culture of chondrocytes *in vitro*(Wen et al., 2023; Zhang et al., 2023). The chemoresistance observed in CS is thought to originate from the microenvironmental features of tumor tissues, primarily the cartilage ECM and hypoxia. As the efficacy of CS treatments is significantly influenced by microenvironmental factors, 3D culture

systems offer a promising avenue for concurrently investigating the response of CS to chemotherapeutic agents and the biological parameters of tumor cells. Analogous to chondrocytes, Pellets represent a key 3D culture method for CS cells, employing centrifugal force to induce spheroid formation through cell-to-cell adhesion to the tube's bottom(Voissiere et al., 2017). Although this technique is straightforward and rapid, the resultant shear stress may potentially damage tumor cells.

CS cell line HT-1080, along with two patient-derived CS cell lines, TP19-S26 and TP19-S115, were cultured in 96-well ULA plates to assess the viability of CS cell sphere formation. Quantitative PCR (qPCR) analysis revealed differential expression levels of CS marker genes (e.g., vascular endothelial growth factor α(VEGFα), hypoxia-inducible factor 1α(HIF 1α), COL2A1, and COL10A1) between 2D-cultured cells, 3D CS spheroids, and primary tumors(Zachos, 2021). These findings suggest that CS spheroids exhibit the biological characteristics of CS and can more accurately replicate the growth pattern and chemotherapeutic resistance observed in patients.

Nevertheless, chondrocyte spheroids with diameters of up to 1.5 mm were unable to preserve their phenotype while simultaneously sustaining volume expansion *in vitro*. Voissiere et al. opted for a nonadherent approach utilizing methylcellulose, which elevates the medium's viscosity and enables CS cells to autonomously aggregate into spheroids in static culture, thus producing their own ECM. Similar to chondrocytes, these CS spheroids manifest as a necrotic core at diameters exceeding 200–300 μm, and the ECM's quality diminishes. This phenomenon may be associated with the impaired diffusion of nutrients and oxygen to cells in the core region, thereby restricting cell growth(Voissiere, et al., 2017). Hypoxia in CS tissues has been shown to be associated with poor prognostic processes, such as cancer cell survival, tumor angiogenesis, invasion, metastasis, radioresistance, and chemoresistance(Chen et al., 2011; Chen et al., 2024). In response to hypoxic stress, CS spheroids cultured in 3D secrete VEGF factors with pro-angiogenic effects. In contrast, spheroids without hypoxic necrotic cores do not secrete VEGF. It has been shown that VEGF expression is upregulated in hypoxic culture conditions and secreted to the outer surface of 3D-cultured spheroids(Terashima et al., 2016).

Analogous to the OS 3D culture method, the utilization of natural or synthetic materials represents an alternative approach for creating 3D CS systems, serving as a scaffold for the assembly of tumor mass cells in CS. Alginate hydrogel, an important biomaterial, significantly contributes to the fabrication of 3D cell models(Radoor et al., 2024). In contrast to other biomaterials like collagen and matrigel, alginate hydrogel displays inert and stable characteristics, making it reproducible for bead formation and well-suited for encapsulation purposes. Alginate hydrogel scaffolds have excellent biocompatibility and can withstand physiological mechanical strains, providing an optimal environment for cell growth as cells are embedded in them. CS cells cultured on these scaffolds can deposit ECM, closely mimicking the growth environment of native CS tissues and accurately reflecting the growth status of CS cells *in vivo*. Moreover, the typically small pore size (5–200 nm) of alginate scaffolds makes them suitable for drug treatment of the encapsulated cells, as small soluble molecules can diffuse freely around the cells, providing an ideal environment in which to test the effects of chemotherapeutic drugs on CS cells(Radoor, et al., 2024). These attributes establish alginate scaffolds as an ideal platform for the 3D cultivation of CS cells and the investigation of drug resistance mechanisms. By utilizing 3D models of three CS cell lines (CH2879, JJ012, SW1353) constructed with alginate beads, researchers have demonstrated that CS cells recapitulate gene expression in the ECM. This model provides a valuable tool for researchers to gain deeper insights into the growth mechanisms of CS cells and offers a promising reference for the development of novel therapeutic strategies(Lhuissier et al., 2017).

3.2 Three-dimensional culture of CS to explore drug resistance

As previously discussed, CS is resistant to conventional chemotherapeutic drugs, which can be explained by their dense tissue structure and the abundant presence of HA in the ECM. The resistance of different CS cell types to chemotherapeutic agents is intricately linked to their capacity for cartilaginous matrix production. Hence, 3D models capable of accurately replicating the *in vivo* characteristics of CS will serve as a crucial tool for investigating the mechanism of CS drug resistance in depth. Elevated ex-

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pression levels of lactate dehydrogenase-A (LDH-A) and mutations in IDH1 and IDH2 are closely associated with the emergence of drug resistance in CS(Walter et al., 2023).

Sapanisertib, an mTOR inhibitor, is considered a potent antiproliferative agent; however, its efficacy against CS in clinical settings has been limited when used alone. Researchers subjected CS spheroids to prolonged sapanisertib treatment and observed that, unlike monolayer-cultured CS cells, the mTOR downstream phosphokinase pS6 remained expressed within the spheroids, indicating the presence of drug resistance(Palubeckaitė et al., 2020). This result is consistent with previous observations in animal models and clinical data. Nevertheless, long-term disease stabilization was noted in CS patients through the combination of mTOR inhibitors with other drugs, including liposomal doxorubicin or inhibitors of epidermal growth factor receptor (EGFR), insulin-like growth factor 1 receptor (IGF1R), and VEGF. The efficacy of this combination may be related to the simultaneous inhibition of multiple signaling pathways, which enhances the therapeutic effect(Trucco et al., 2018).

P-glycoprotein (P-gp), encoded by the multidrug resistance (MDR1) gene, is a member of the ATP-binding cassette (ABC) family of transporters, which utilize the energy generated by ATP hydrolysis to transport various substrates, including anticancer drugs. P-gp reduces the lethality of a variety of antitumor drugs for tumor cells, including anthracyclines (e.g., doxorubicin (DXR)), vinca-alkaloids (e.g., vincristine), podophyllotoxins (e.g., etoposide), and taxanes (e.g., taxol)(Zhang et al., 2021; Halder et al., 2022). However, resistance of CS cells to DXR was not associated with overexpression of P-gp, as seen from the analysis of MDR1 gene expression in CS cells (SW1353) cultured in monolayer and suspension droplet methods.

Salinomycin (SAL), an ionophore, has been identified as a promising drug for CS treatment through spheroid-based 3D cell cultures. SAL exerts its effects on 3D-CS spheroids by activating cysteine asparaginase, leading to apoptosis induction. Reduced viability and enhanced DXR nuclear localization demonstrated that SAL also enhanced DXR cytotoxicity in 3D-CS spheroids at sublethal doses. Therefore, SAL or its derivatives can be considered a new option for the treatment of CS(Perut et al., 2018).

Talazoparib, a poly (ADP-ribose) polymerase (PARP) inhibitor, demonstrated remarkable inhibitory effects on CS cell lines, increasing their sensitivity to conventional chemotherapeutic agents (e.g., temozolomide) or radiation therapy, regardless of their IDH mutation status(Venneker et al., 2019). To further understand the efficacy of PARP inhibitors in decreasing CS aggressiveness, researchers chose three widely studied CS cell lines—CH2879 (IDH wild-type), JJ012 (IDH1 mutant), and SW1353(IDH2 mutant). These cell lines were cultured into spheroids using alginate as a scaffold and exposed to various concentrations of talazoparib, temozolomide, or a combination of both. The results showed that long-term PARP inhibition was more effective than short-term treatment, and only one CS cell line showed sensitivity to the combination of PARP inhibition and radiotherapy(Palubeckaite et al., 2023). Collectively, these findings suggest the importance of focusing future animal studies on long-term PARP inhibition and highlight the potential efficacy of combining temozolomide with talazoparib compared with radiotherapy. In addition, the talazoparib and temozolomide combination effectively reduced the viability and growth of CS spheroids, regardless of IDH mutation status, signifying a promising avenue for further comprehensive investigation of this chemotherapy combination in animal models of CS.

Patient-derived organoids (PDOs) possess the genotypic characteristics of tumors from patients, enabling high-throughput and rapid *in vitro* clinical drug testing. The tissues used to create PDOs were obtained through serial biopsies performed by clinicians on tumor patients at multiple regional sites (including at baseline, best response, and disease progression). Researchers screened 55 drugs in Phase I-III clinical trials or clinical practice using PDOs. By comparing PDO drug responses with patient responses in PDO-based orthotopic mouse tumor xenografts and clinical trials, the researchers concluded that PDOs can replicate patient heterogeneity and serve as a valuable tool for optimizing specific drug therapies(Vlachogiannis et al., 2018).

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4 Ewing sarcoma (ES)

Ewing sarcoma (ES) is a highly aggressive malignancy that primarily affects the bones and soft tissues of children and young adults. The clinical presentation of ES is often insidious, with patients experiencing mild, intermittent pain that may intensify during the night or with physical activity. In some cases, the only discernible sign of the disease is the presence of a firm mass upon physical examination. Elevated serum levels of lactate dehydrogenase have been associated with tumor burden in ES patients, potentially providing valuable diagnostic and prognostic information. Despite ES being highly sensitive to radiotherapy, which frequently results in rapid tumor shrinkage, the long-term prognosis for patients remains dismal. Therefore, early detection and the implementation of effective treatment strategies are critical for improving patient survival and quality of life. To further advance our understanding of ES biology and develop novel therapeutic approaches, future research efforts should focus on establishing an *in vitro* model that accurately recapitulates the complex microenvironment observed *in vivo*(Riggi et al., 2021; Zöllner et al., 2021).

4.1 Three-dimensinal culture for predicting therapeutic targets for ES

Current targeted therapies for ES primarily focus on downstream signaling cascades triggered by EWS-FLI1 activity (e.g., IGF1/IGF-1R, TGF-β, Hedgehog/GLI, Wnt/β-catenin, and Notch/p53) or on ES cell-dependent aspects of the surrounding tumor stroma(Smith et al., 2006; Hawkins et al., 2020; Dupuy et al., 2023). Suspension culture of ES cells induces an immediate serum-independent activation of ERK1/2 and AKT pathways, indicating that ES spheroids more closely resemble primary tumors in terms of cell–cell junctions, proliferation indices, and biochemical metabolism(Lawlor et al., 2002). In addition, the expression of cyclin D1 protein, a critical cell cycle regulator, was completely blocked prior to the formation of stable multicellular spheroids, implying that cell–cell adhesion is essential for the proliferation of ES cells. Pharmacological inhibition of PI3K significantly reduced the proliferation of ES cells, and the expression level of cyclin D1 was correlated with the activated expression of AKT. Therefore, the PI3K-AKT pathway plays a crucial role in ES cell proliferation in both *in vitro* and *in vivo* settings, and studying ES spheroids may provide a more accurate representation of tumor cell growth *in vivo*(Lawlor, et al., 2002).

IGF-1R and its downstream pathways play an important role in the pathogenesis and development of ES(Lamhamedi-Cherradi et al., 2016). Culturing ES cells on electrostatically spun poly(ε-caprolactone) (PCL)3D scaffolds in a flow perfusion bioreactor was able to mimic the mechanical stimulation of the bone microenvironment. This mechanical stimulation notably enhanced the proliferation of ES cells cultured in 3D and upregulated the expression of IGF1, indicating a potential role for mechanical cues in the tumorigenesis and development of ES(Santoro et al., 2015; Santoro et al., 2016). The findings of this study not only deepen our understanding of the mechanisms of tumor growth and development but also provide an important theoretical basis for the development of more effective tumor treatment strategies in the future. Therefore, when constructing a representative model of pediatric ES characterized by IGF1 upregulation and dysregulation, flow perfusion culture more accurately recapitulates the *in vivo* behavior of ES compared to static culture conditions(Santoro, et al., 2015; Kirk et al., 2020).

4.2 Three-dimensional culture for exploring ES microenvironments

The TME is a crucial part of understanding ES biology and its response to treatment. Accumulating evidence indicates that stromal cells significantly influence the generation of the tumor immune environment by inhibiting peripheral blood monocyte proliferation, compromising natural killer cell cytotoxicity, mediating endothelial barriers to lymphocyte infiltration, and disrupting the effectiveness of checkpoint inhibitor therapies in T cells(Feig et al., 2013; Santoro, et al., 2016). Culturing ES cells in the PCL-ECM construct environment mimics their morphology, exhibiting a clustered, rounded cell phenotype, whereas, in the PCL environment, cells tend to grow along fibers in smaller clusters or as single cells. In addition, the proliferation rate of ES was accelerated within 5 days after the addition of ECM and mineral components to the PCL environment, suggesting that these constructs may be useful for investigating the mechanism of ES tumor multiplication.

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Interestingly, the increased aggregation and proliferation of ES tumors may not be due to the bioactive components in the PCL-ECM microenvironment but rather to topographical differences between the two studied 3D environments(Molina et al., 2020). ES tumors promote immune evasion and metastasis by polarizing macrophages towards a TAM phenotype, while TAM in the TEM promotes immune evasion and metastasis by promoting angiogenesis (enhancing tumor vasculature) and osteoclastogenesis (contributing to bone destruction), thereby promoting tumor proliferation and metastasis(Feig, et al., 2013).

Domenici's team used an innovative method to encapsulate PDX-derived ES cell spheroids in alginate for 3D cell culture, which maintained ES spheroid cell viability and proliferation for at least one month. The ES cells exhibited high proliferative and metabolic activity while retaining the typical EWSR1-FLI1 chromosomal translocation(Domenici et al., 2021). Preserving the existence of EWSR1-FLI1 serves a dual purpose: probing the oncogenic significance of these proteins in ES advancement and assessing the potential pharmacological utility of EWSR1-FLI1-specific inhibitors(Erkizan et al., 2010; Meyers et al., 2024). ES 3D cultures can be used for drug sensitivity assays, yielding results similar to those of primary PDX. This novel 3D cell culture method involving ES-PDX-derived cells represents a suitable model for studying the pathobiology of ES and could facilitate the development of novel drugs against this disease, complementing PDX studies. Unlike CS spheroids, ES 3D spheroids exhibited no evidence of hypoxia, as evidenced by the absence of elevated mRNA expression of the HIF-1α transcriptional target CAIX and the lack of a detectable hypoxic environment using specific hypoxic fluorescent dyes(Krieg et al., 2000). Hypoxic cores within cancer cell spheres were only observed when the sphere diameter exceeded 400 μm. Again, these findings support the importance of 3D culture systems in recapitulating the complex TME and provide valuable insights into the mechanisms driving ES pathogenesis and progression.

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5 Future perspective and challenges

The complex progression and metastasis of bone tumors are orchestrated by a multitude of factors, with interactions within the physiological, structural, and biochemical TME. As scientific research has progressed, a concerning trend has emerged: numerous therapies that showed promise in 2D cultures have consistently failed in clinical trials, highlighting the inherent limitations of 2D cultures in accurately recapitulating the intrinsic characteristics of tumors(Kim and Lee, 2023). While significant advancements have been made in developing 3D cancer models that mimic soft tissue environments, such as those found in colorectal and pancreatic cancers, the field of tissue-engineered 3D models that faithfully replicate the dynamics of primary and metastatic bone cancers remains largely unexplored and is still in its infancy.

In this review, 3D culture methodologies that emulate TME have emerged as a novel vantage point in the study of bone tumors (**Fig. 2**). Notably, 3D culture systems that employ natural or synthetic scaffolds to mimic the bone microenvironment have shown great potential in investigating tumor invasion, metastasis, angiogenesis, and the development of antitumor drugs. The advent of cutting-edge technologies, such as microfluidics, bioprinting, and the integration of perfusion systems, has enabled the creation of customized 3D systems. These advancements not only replicate the natural configuration of bone tumors, encompassing cell–cell or tissue interactions, cell adhesion, proliferation and migration, and ECM structure and composition, but also simulate physiological parameters, such as hypoxia, and mechanical factors, including shear stress and mechanical forces. By providing a more authentic research environment, these 3D models offer unprecedented opportunities to unravel the complexities of bone tumor biology and develop novel therapeutic strategies. As the field continues to develop, we anticipate that the integration of 3D models with other cutting-edge technologies, such as organ-on-a-chip systems, single-cell sequencing, and machine learning algorithms, will revolutionize our understanding of bone tumors and pave the way for the development of more effective and personalized therapies(Xu et al., 2022; Truong et al., 2024). However, the development and application

of these advanced 3D models are not without challenges. Three-dimensional tissue engineering strategies for malignant bone tumors aim to develop cost-effective *in vitro* experimental models and implantable *in vivo* models by integrating biocompatible scaffolds, invasive bone tumor cells, and bioactive agents. However, these culture strategies have several limitations, including (1) scaffolds designed for mature rather than developing tissues, (2) the delivery of a single morphogen, (3) interference with cell–cell interactions, (4) asynchronous degradation with new tissue formation in 3D culture, and (5) potential immunogenic or cytotoxic effects of scaffolds or their degradation byproducts. Despite the numerous advantages of existing 3D tumor models, their inherent properties also inevitably limit their clinical applications. The complexity of the bone TME, which encompasses a wide range of cell types, ECM components, and signaling molecules, poses significant technical hurdles in creating truly representative 3D models. Additionally, the lack of standardized protocols and the high cost associated with the development and maintenance of these models may hinder their widespread adoption. Furthermore, the translation of findings from 3D models to clinical practice requires rigorous validation and the establishment of robust preclinical testing pipelines. Consequently, further research is necessary to address these issues in preparation and optimize their performance for clinical application to bone tumors.

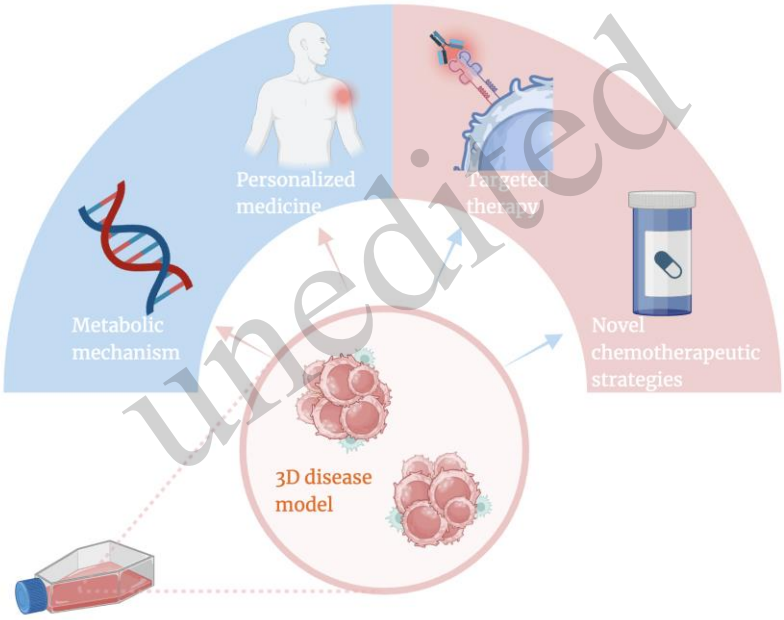

Fig. 2 Schematic overview showing the wide range of applications of malignant bone tumor models constructed with 3D culture technology in oncology research, clinical treatment, and other areas. Created in BioRender.com.

6 Conclusions

This comprehensive review provides an in-depth analysis of the current state-of-the-art in utilizing *in vitro* 3D culture models to study malignant bone tumors, elucidating their role in investigating the immune microenvironment of bone tumors, refining culture techniques, and understanding resistance mechanisms to chemotherapeutic agents. These investigations have not only propelled the advancement of fundamental cancer research but have also paved the way for preclinical investigations and the development of innovative chemotherapeutic strategies. Techniques such as microfluidics, 3D bioprinting, hydrogel engineering, and sphere or organoid technologies hold great promise in creating more physiologically representative 3D models of bone tumors. While the focus is typically on OS, CS, and ES, the rarity of bone cancer is acknowledged. Hopefully, the principles

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discussed in this review can be broadly applied to other sarcoma subtypes or bone metastatic cancers, offering a holistic approach to advancing our understanding and treatment of these malignancies.

Data availability statement

Data will be made available on request.

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Author contributions

Z.H. wrote the manuscript. H.H. and J.F. contributed to the literature collection, H.L. contributed to graphic generation. H.W. and X.Y. conceived the topic and revised the manuscript, contributed to the funding acquisition.

Compliance with ethics guidelines

Zhengcheng HE, Haitao HUANG, Jiale FANG, Huiping LIU, Xudong YAO and Hongwei WU declare no conflicts of interests.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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