THREE-DIMENSIONAL CULTURE SCAFFOLDS FOR OSTEOSARCOMA CANCER STEM CELLS

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Abstract

Cancer stem cells (CSCs) are a subpopulation of tumour cells that are highly tumorigenic with self-renewing potential. In osteosarcoma, these cells are responsible for drug resistance and cancer relapse. Studying CSCs in vitro can provide a better development of therapeutic strategies by understanding the mechanism of tumorigenesis and chemoresistance in osteosarcoma. Cell culture plays a crucial role in cancer research, stem cell studies, and drug discovery. While two-dimensional (2D) methods are commonly used for cell culturing, recent advancements in three-dimensional (3D) techniques offer promising opportunities for conducting complex experiments. With 3D cell culture, the cellular environment can be manipulated to closely mimic in vivo conditions, resulting in more accurate data about cell-to-cell interactions and tumour characteristics. Various scaffold-based techniques using (1) natural polymers such as hydrogel, collagen type I, agar gel, Matrigel, alginate, bacterial cellulose, hyaluronic acid, and (2) synthetic polymers such as polyethylene glycol diacrylate (PEGDA) and poly (2-hydroxyethyl methacrylate) (pHEMA) offer unique advantages and applications for studying osteosarcoma CSCs. Scaffold-free techniques such as ultra-low binding plates and hanging drop are also used to culture osteosarcoma CSCs. This review article describes various 3D culture methods used in forming osteosarcoma CSC spheroids and the expression of stemness markers.

Keywords: 3D Culture, Osteosarcoma CSCs, Scaffold, Stemness

Introduction

Osteosarcoma is a primary bone tumour in children and adolescents and the third most common in adults, following chondrosarcoma and chordoma. The global incidence is 3.1 per million per year (1). This neoplasm is characterised by bimodal age distribution, with the first peak between ages 10 and 14 and the second peak over age 65 (2). The first peak occurs in adolescents due to rapid linear bone growth localised in the metaphysis of long bones, particularly the distal femur, proximal tibia, and proximal humerus (3). Meanwhile, the second peak in the elderly is associated with Paget's disease and excessive bone resorption (4).

The pathophysiology of osteosarcoma involves a complex interplay between genetic and environmental factors. Malignant proliferation of mesenchymal cells produces immature bone or osteoid tissue. It can be classified into subtypes based on the tumour's features and stromal differentiation, either osteoblastic, chondroblastic, fibroblastic, small-cell, or extraskeletal. Looking at the histology appearance, malignancy of the bone can be classified into high-grade, intermediate-grade and low-grade. Osteosarcoma is the most frequent type of high-grade bone malignancy growing intramedullary (2).

The current regimen use to treat osteosarcoma combines surgery with multimodal preoperative and postoperative chemotherapy using more than two cytotoxic agents such

as doxorubicin, cisplatin, ifosfamide, and methotrexate (4). Despite therapeutic advances, the 5-year survival rate remains stagnant at 70% for localised osteosarcoma and drastically decreases to 20-30% for patients with metastatic (5). The effect of chemotherapy is dampened due to the heterogeneous nature of the tumour, resulting in chemoresistance and metastasis. A small subgroup of cells within a tumour recognised as cancer stem cells is thought to cause inherent resistance and can self-renew and differentiate into different types of cancer cells. A subset of CSC that survives after successful cancer therapy can promote relapse and greater invasiveness. Up to the present, researchers have identified cancer stem cells (CSCs) in acute myeloid leukaemia, pancreatic, lung, ovarian and breast carcinomas, osteosarcoma, and glioblastoma (6, 7).

Osteosarcoma cancer stem cells

Cancer stem cells (CSCs) are tumour-initiating cells that are derived from either adult stem cells, progenitor cells that are mutated, or from differentiated cancer cells that acquire stem-like properties through dedifferentiation (8). Osteosarcoma-containing cells with CSC characteristics were first discovered by Gibbs et al. (9) when grown in low attachment plates supplemented with serum-free semi-solid N2 medium together with epidermal and fibroblast growth factors. CSCs in osteosarcoma were isolated based on their ability to form sarcospheres in an anchorage-independent and serum-starved growth condition (10). This small subpopulation of cells was sorted and isolated according to the expression of specific markers related to stemness.

Markers such as $CD133^+$, $CD271^+$, $CD117^+$, and $STRO1^+$ sorted from osteosarcoma have been found to demonstrate CSC-like features. Other methods used to isolate osteosarcoma CSCs include the ability of the subpopulation to exclude certain fluorescent dyes, the enzymatic activity of aldehyde dehydrogenase 1 (ALDH1), and tracking the expression of pluripotency-associated genes, such as OCT3/4, Nanog, and SOX. The human osteosarcoma cell lines, Saos-2, OS99-1, MG-63, and Hu09 can form spheroids which express OCT4 and Nanog mRNA (11, 12). Inhibition of stemness markers Nanog, CD133, ALDH1, OCT3, and SOX2 sensitises cancer stem cells to chemotherapy (13, 14).

In experiments using human osteosarcoma cell lines, Saos-2, OS99-1, MG-63, and Hu09, the formation of spheroids was reproducible when passaged multiple times and produced adherent cell cultures when supplemented with a standard growth medium. The MG-63 spheroids were less sensitive to cisplatin and doxorubicin than adherent cells, suggesting the spheroids may have developed chemoresistance. This is likely due to the increased expression of DNA mismatch repair enzymes MLH1 and MSH2 (15). Similarly, CSCs derived from osteosarcoma can self-renew in repeated transplantation trials and are associated with tumour metastasis and treatment resistance (16). These CSCs exhibited high levels of ABC transporters mRNA, including ABCA1, ABCB2, ABCB2, and ABCG2, which are associated with treatment resistance. The CSCs also expressed high levels of CD248, CD133, OCT3/4, and Nanog mRNA, responsible for CSC self-renewal (16).

Aside from the ability to repair DNA and express the drug transporter, the other mechanism causing CSCs to become chemoresistance is their ability to induce cell cycle arrest, particularly in the quiescent state (G_0/G_1 phase) (17). Being quiescent, CSCs could escape chemotherapeutic treatments aimed at attacking rapidly dividing cancer cells. This gives CSCs and cancer cells an advantage in survival and promoting cancer relapse after chemotherapy (18). Additionally, the lack of predictive 3D in vitro models to simulate osteosarcoma heterogeneity and complexity contributes to treatment failure (19). Isolating CSCs and developing cell culture techniques for CSCs is essential to improve the understanding of osteosarcoma biology and pathogenesis.

Two-dimensional (2D) versus three-dimensional (3D) cell culture

Since the early 1900s, the two-dimensional (2D) cell culture has been the principal method for cell culture. It offers the benefits of easy and low-cost maintenance of cultures and enables functional tests to be performed efficiently. While this approach is critical for research purposes, it has several shortcomings, as 2D models do not accurately mimic tissue cells or tumours in vitro (20). The 2D culture technique lacks representation of cell-cell and cell-extracellular environment interactions within the tumour mass. These interactions play a critical role in cell differentiation, proliferation, expression of genes and proteins, drug metabolism, responsiveness to stimuli, and other cellular functions (20, 21). When cells are isolated from tissue and grown on 2D cultures, the morphology of the cell is transformed during cell division and some of the phenotype is lost (22). This affects the cell function, organisation of the organelles inside the cell, cell signalling, and secretion (23).

Alternatively, monolayer cells in culture are more accessible to the medium containing nutrients, oxygen, and metabolites. However, tumour cells in vivo lack this access due to the natural architecture of the tumour mass (24). In numerous 2D culture experiments, the interaction between various cell types is not considered, and most cultures involve a single cell type. While 2D co-cultures address some of these limitations, they still do not adequately replicate the cellular functions observed within a tissue.

Although research has been conducted on new drugs using 2D approaches, their effectiveness differs from in vivo studies. This can be attributed to the low translatability resulting from the inconsistent use of 2D models (25, 26). Due to the numerous drawbacks of 2D systems, exploring alternative culture models that can more accurately simulate a natural microenvironment of tumour mass, such as 3D culture systems, became necessary.

Studies focusing on morphology, proliferation, differentiation, drug metabolism, protein synthesis, angiogenesis, migration, invasion, and metastasis have demonstrated enhancements using 3D cell culture (27). This is possible as 3D cultures provide better similarities of the cell-extracellular matrix and intercellular signalling interactions. Currently, 3D cell culture has offered a new opportunity in tissue engineering and provides a better understanding of the disease and has been proposed to be the practical model for targeting cancer and cancer stem cells in the development of better therapies for osteosarcoma (28).

Compared to cultures in 2D, $CD133^+$ cells cultured in 3D osteosarcoma spheroids exhibited more extended maintenance of stem cell phenotype, resulting in 5.88-, 4.14-, 6.96-, and 1.68-fold higher mRNA expressions for Sox2, OCT3/4, Nanog, and Nestin, respectively (29). The study concluded that CSC-based tumoroids are promising 3D tumour models that can bridge the gap between conventional 2D in vitro cultures and in vivo animal experiments for cancer research. This is due to the longer maintenance of stem cell phenotype observed in 3D cultures, which can enhance the relevance of screening and targeting efficiency in drug testing.

Generally, mouse models are used to test new drugs and treatment approaches, particularly in cancer research. However, the advancement of 3D culturing techniques allows researchers to construct and conduct drug treatment tests, reducing the need for animal models (30).

3D culture scaffolds in osteosarcoma cancer stem cell (CSC) studies

The first 3D culture was conducted using soft agar solution in the 1970s by Hamburger and Salmon (31). Now, 3D cultures can be prepared using (i) scaffold-based, (ii) scaffold-free, and (iii) non-adherent plates for suspension cultures (32). Other 3D culture techniques include microfluidic devices and bioprinting. Microfluidic devices use microchannels and chambers to create a controlled microenvironment for cells to grow in 3D. Bioprinting involves using a 3D printer to create structures from biological materials such as cells and extracellular matrix components, allowing for the precise placement of cells and materials to form 3D structures (33). This review will further summarise some 3D culture scaffolds used in osteosarcoma CSCs experiments.

Scaffold-based 3D cultures using natural polymers

Scaffold-based 3D culture techniques involve using a scaffold, which provides a physical and biochemical environment that mimics the natural extracellular matrix of tissues and organs to support the growth and organisation of cells. Cells are seeded onto, or into the scaffold, and over time, they interact with the scaffold and with each other to form 3D structures that resemble tissues and organs in vivo (33).

Various scaffold-based methods offer numerous benefits, including support systems such as hydrogels, hydrophilic glass fibres, organoids, and polymeric material. One of the distinctive advantages of scaffolds such as hydrogel is their ability to imitate the extracellular matrix (ECM) and permit soluble agents like growth factors and cytokines to permeate the gel-like tissue (34). In addition, hydrogels are adaptable and can be employed to produce spheroids through various preparation techniques according to the requirement of the experiment. Hydrogels can either be natural or synthetic, with natural gels typically consisting of natural polymers such as hyaluronic acid, fibrinogen, collagen, gelatin, Matrigel, alginate, and chitosan.

Collagen Type I

Researchers created a 3D tissue-engineered model of osteosarcoma using a bone-like scaffold consisting of hydroxyapatite nanoparticles and collagen type I to explore the potential of cold atmospheric plasma (CAP) as an anti-cancer therapy by investigating its impact on oxidative stress (35). The study findings indicate that the 3D environment not only protected cells against the lethality caused by Plasma-Activated Ringer's Solution (PAR) through scavenging and reducing the amount of reactive oxygen and nitrogen species generated by CAP but also promoted the stemness phenotype of osteosarcoma cells. Results from RT-PCR showed a significant increase in the expression of genes associated with stemness and cancer stem cell phenotype in osteosarcoma, such as SOX2, OCT3/4, and NANOG (35). The researchers suggest that using collagen type I and hydroxyapatite nanoparticles as a scaffold is useful in developing biomimetic models suitable for studying anti-cancer therapies in bone cancer and metastasis.

In another study, osteosarcoma CSCs from two different cell lines, MG-63 and SAOS-2, were analysed (36). The findings demonstrated that using a scaffold composed of collagen type I and hydroxyapatite created a tumour microenvironment that more closely mimicked the behaviour of these cells. After ten days of 3D culture, the stemness markers OCT4, NANOG, and SOX-2 mRNA were quantified by qPCR. The expression of NANOG mRNA in MG-63 cells was significantly upregulated by 4.6-fold in a scaffold composed of collagen type I and hydroxyapatite compared to scaffold-free culture. Meanwhile, in SAOS-2 sarcospheres, OCT4 mRNA expression was increased by 9.1-folds as compared to scaffold-free culture. Both MG-63 and SAOS-2 cell lines demonstrated an increase in the expression of SOX-2 mRNA, however, showed no statistically significant differences. The qualitative evaluation of OCT4 and SOX-2 using immunofluorescence staining confirmed the expression of these stemness markers in MG-63 and SAOS-2 sarcospheres.

The significant elevation of OCT4, NANOG, and SOX-2 gene expression in the proposed 3D models serves as evidence that the presence of a 3D biomimetic scaffold, which mimics the nanostructure and physicochemical properties of the natural environment, induces a more pronounced

stem phenotype in 3D sarcospheres as opposed to those cultured without scaffolds. These genes are commonly utilised as stemness markers due to their crucial role in preserving cancer stem cell pluripotency and self-renewal properties. These genes are commonly associated with the signalling between CSCs and the tumour stem niche, forming a sophisticated intercellular network that regulates stemness and the fate of CSCs. The findings also demonstrated higher expression of NOTCH-1 and HIF-1α in collagen type I and hydroxyapatite scaffold compared to the scaffold-free model (35). This confirms that the 3D mimetic environment plays an active role in preserving the phenotype of cancer stem cells (CSCs).

Agar gel

A study conducted by Ozturk et al. (29) aimed to develop a tumoroid model using CSCs for high-throughput drug screening, drug targeting and personalised medicine. Therefore, $CD133^+$ cells were isolated from SAOS-2 osteosarcoma cell line using magnetic-activated cell sorting. These cells were cultured in agar gels produced by the 3D Petri Dish method to evaluate tumoroid formation ability. The findings showed that $CD133^+$ cells, $CD133^-$, and SAOS-2 cells could form 3D tumoroids, but $CD133^+$ cells took longer to self-assemble. $CD133^+$ cells were located randomly within tumoroids with high cell viability. In comparison to 2D cultures, the mRNA expressions for Sox2, OCT3/4, Nanog, and Nestin were respectively 4.14, 6.95, 5.88, and 1.68 times higher in $CD133^+$ cells cultured in 3D tumoroids (29). Furthermore, the immunostaining results indicated that $CD133^+$ cells expressed CD133, OCT3/4, and the cell proliferation marker Ki-67. This indicates that the stem cell phenotype is maintained for a longer period in the 3D culture.

Matrigel

Matrigel is a type of extracellular matrix (ECM) protein mixture commonly used as a substrate for cell culture experiments. It is derived from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma and contains various ECM proteins such as laminin, collagen, and glycosaminoglycans. Matrigel is often used in cell culture experiments as a three-dimensional (3D) matrix that can provide a more physiologically relevant environment for cells to grow and interact with each other. It has also been shown to support the growth and differentiation of stem cells and cancer cells and can be used to study cell behaviour in vitro.

A study by Di Fiore et al. (37) aimed to establish an in vivo model for human osteosarcoma CSCs. The researchers subcutaneously injected both 3AB-OS osteosarcoma CSCs (isolated from MG63 cells) and parental MG-63 human osteosarcoma cells into athymic mice, with and without Matrigel. The findings showed MG-63 cells lacked tumour-forming ability in vivo, while the 3AB-osteosarcoma CSC cells were highly tumorigenic, especially with Matrigel. The stemness features of 3AB-osteosarcoma CSC cells were preserved after being engrafted into nude mice. During the first few weeks of engraftment, stemness-related genes such as CD133, OCT3/4, SOX2, NANOG, HMGA2, h-TERT, Nucleostemin, Nestin, Nucleostemin, ABCG2, and Lin28B were highly expressed, regardless of Matrigel presence. However, as time passed, the levels of these markers decreased significantly, particularly in the presence of Matrigel in xenografts. During the early weeks of engraftment, both tumour growth rate and proliferation index analysed using Ki-67 and PCNA were increased but significantly declined over time. The use of Matrigel was found to accelerate these changes. These findings suggest that Matrigel may influence tumour cell behaviour and facilitate the gradual loss of stemness by interacting with the microenvironment.

Alginate

Alginate is a natural polysaccharide derived from brown seaweed that is commonly used in tissue engineering and regenerative medicine to create scaffolds or support structures for cell growth and tissue regeneration (38). Alginate scaffolds are porous structures that allow for the diffusion of nutrients, oxygen, and waste products to and from the cells within the scaffold. The scaffold's porosity can be controlled by altering the concentration of the alginate solution, the crosslinking agent used, and the fabrication method. This type of scaffold has several advantages over other biomaterials, including their low toxicity, ease of fabrication, and ability to form gels under physiological conditions (39).

A study conducted by Zhou et al. (40) isolated and identified CSCs from human osteosarcoma using serum-free alginate 3D culture combined with anti-cancer drugs. A single-cell suspension of primary cells from human osteosarcoma was prepared by digesting them with trypsin and homogeneously mix into 1.2% alginate gel and epirubicin to enrich CSCs. Most of the cells were killed by epirubicin, but some survived and formed single-cell cloning spheres after 7 to 10 days in culture. The spheres stained positive for OCT3/4 and NANOG. Most of these positive cells were concentrated in the core of the sphere. These spheres were transplanted into BALB/c mice and were able to form osteosarcoma in mice, suggesting that they had properties of stem cells, resistance to anti-cancer drugs, and tumorigenicity in vivo.

Bacterial cellulose

Bacterial cellulose scaffold is a type of biomaterial made from bacterial cellulose fibres synthesised by certain strains of bacteria, typically from the Acetobacter genus (41). These scaffolds are biocompatible and have unique physical and mechanical properties, such as high porosity and mechanical strength, making them suitable for tissue engineering and regenerative medicine applications. Bacterial cellulose scaffolds can support structure for growing cells, as they can mimic the extracellular matrix of natural tissues and provide a microenvironment that promotes cell growth and differentiation.

A study examining the effects of hypoxia on SaOS-2 osteosarcoma cell viability, morphology, and stemness was performed using bacterial cellulose scaffolds (42). By mimicking tumour structures and replicating in vivo conditions, the 3D bacterial cellulose scaffolds could support osteosarcoma tissue microarchitecture. The findings demonstrated that hypoxia did not adversely impact the viability of osteosarcoma cancer stem cells and that the subpopulation of osteosarcoma cells maintained their stemness and pluripotency.

Hyaluronic acid

The hyaluronic acid (HA) scaffold is a three-dimensional structure composed of hyaluronic acid molecules that can mimic the extracellular matrix of the native tissue. The scaffold can support and guide the growth and differentiation of cells and facilitate tissue repair and regeneration. HA scaffolds can be produced by various methods, such as crosslinking with chemical or physical agents, electrospinning, and 3D printing. Despite their biocompatibility, biodegradability, and ability to support cellular activities, HA scaffolds have some disadvantages. This includes weak mechanical properties and fast degradation in vivo. However, it is possible to address these issues through chemical modification or crosslinking techniques, which can improve its mechanical properties, degradation rate, solubility, viscosity, and biological characteristics (43)

A study conducted by Lin et al. (44) fabricated a bioactive scaffold using osteosarcoma cells and gelatine methacrylamide (GelMA)/hyaluronic acid methacrylate (HAMA) hydrogel to model the interaction between three different osteosarcoma cell lines (HOS, U2-OS, and 143B) and the extracellular matrix. The HOS cells were cultured in ultra-low attachment culture dishes and supplemented with N2, hEGF, and bFGF growth factors to recover stem cell properties and promote the growth of cell spheroids. Meanwhile, all three-osteosarcoma cell lines cultured with GelMA/HAMA to form bioprinted 3D models were also cultured with medium containing the growth factors. The addition of these growth factors did not produce any significant differences. This shows that GelMA/HAMA scaffold used in bioprinting could support the survival and growth of osteosarcoma cells, demonstrating excellent biocompatibility.

Scaffold-based 3D cultures using synthetic polymers

Synthetic scaffold polymers are artificially created materials designed to support and structure cells and tissues in various biomedical applications, including tissue engineering, regenerative medicine, and drug delivery. These polymers typically comprise biocompatible and biodegradable materials, such as polyesters, polyamides, or polyurethanes. They can be engineered to have specific physical and chemical properties that facilitate cell growth and tissue formation.

Synthetic polymers are preferred over natural polymers to produce tissue-engineering scaffolds due to their consistent physical and chemical characteristics. These polymers create a three-dimensional microenvironment that mimics the extracellular matrix (ECM) of natural tissues, which can promote cell adhesion, migration, proliferation, and differentiation. They have become a crucial alternative for fabricating hydrogel tissue-engineering scaffolds with the ability to tailor synthetic polymers regarding block structures, molecular weights, mechanical strength, and biodegradability. The most commonly used synthetic scaffolds are made from synthetic materials such as poly (ethylene glycol) (PEG), poly(lactic-co-glycolic acid) (PLGA), and poly(caprolactone) (PCL) (45-47)

Polyethylene glycol diacrylate (PEGDA) hydrogel

Polyethylene glycol diacrylate (PEGDA) is a hydrogel widely used as a cell culture scaffold due to its biocompatibility and tunable mechanical properties. The cross-linking of PEGDA monomers forms PEGDA hydrogels with a photoinitiator and UV light, which allows for creating of 3D structures with precise control over porosity and stiffness (48).

The use of PEGDA hydrogels as cell culture scaffolds offers several advantages. Firstly, the 3D structure of the hydrogel allows for the formation of cell-cell and cell-matrix interactions that more closely mimic the in vivo environment. This can improve cell differentiation, proliferation, and migration. Additionally, PEGDA hydrogels can be functionalised with bioactive molecules, such as growth factors or extracellular matrix proteins, to enhance cell behaviour. PEGDA hydrogels can also be designed with specific mechanical properties to match those of the modelled tissue. For example, the stiffness of the hydrogel can be tuned to match that of soft tissue (for example, brain) or stiff tissue (for example, bone), which can improve cell differentiation and tissue formation.

Using the synthetic scaffold PEGDA hydrogel, Jabbari et al. (49) demonstrated PEGDA matrix stiffness in maintaining CSC behaviour in osteosarcoma U2OS cell lines along with other cancer cell lines. The findings demonstrated that the optimum gel modulus for tumorsphere growth and expression of CSC markers varied for different cancer cell types; 5 kPa for MCF7 and MDA231 breast cancer cells, 25 kPa for HCT116 and AGS gastrointestinal cells, and 50 kPa for U2OS osteosarcoma cells. The expression of CSC markers for cancer cells encapsulated in PEGDA gel was consistent with cell densities, tumorsphere sizes, and number densities.

In all culture points, the number of cells in the 3D CSC group was significantly lower than that of the 2D groups for all cancer cell lines. This is probably due to the restricted cell growth in the non-adherent PEGDA gel, which was limited to the stem cell subpopulation. However, the expression of CSC markers CD44, EGFR, and ABCG2 did not increase over time. The expression levels of these CSC markers were significantly higher in 3D CSC groups than in 2D CSC groups.

These results indicated that the elevated expression of CSC markers in the 3D-CSC group was attributable to the cancer cells being encapsulated in the PEGDA gel rather than a change in the culture medium.

Poly-HEMA

The synthetic hydrogel known as poly (2-hydroxyethyl methacrylate) (pHEMA) is produced by polymerising 2-hydroxyethyl methacrylate (HEMA) monomers in situ (50). It is commonly used in cell culture as a substrate or coating material for cell growth. The polymer is hydrophilic and has a flexible monomer that can form a hydrogel network. The hydrogel properties of pHEMA can be modified by changing the degree of polymerisation, crosslinking density, and other parameters during synthesis. pHEMA is often used as a substrate for culturing cells in 2D or 3D cultures, as it allows cells to adhere, proliferate, and differentiate in a controlled manner. It can also be used as a coating material for surfaces such as microplates, glass slides, and other labware to enhance cell adhesion and promote cell growth.

A study conducted by Martins-Neves et al. (51) used poly-HEMA-coated plates to identify and characterise the CSCs population from human MNNG/HOS osteosarcoma cell line and their responsiveness towards chemo- and radiotherapy. The isolated population of CSCs exhibited properties similar to stem cells, including the formation of osteosarcoma spheroids using poly-HEMA-coated plates, expression of mesenchymal stem cells surface markers CD73, CD90, and CD103. Additionally, the population expressed OCT4 and NANOG, responsible for maintaining self-renewal and pluripotency. These CSCs were able to self-renew and generate differentiated progeny through serial passages under selective culture conditions.

The findings demonstrated that spherical clones of CSCs displayed higher chemo- and radioresistance compared to parental cells, which was attributed to the increase in ABC transporters, such as PgP and BCRP pump, responsible for mediating drug efflux of drugs like doxorubicin, cisplatin, and methotrexate. Furthermore, the study showed that the drug resistance of osteosarcoma CSCs increased through several mechanisms, such as their ability to enter a quiescent state, lower cellular division rates, and reduced energy requirements. Additionally, they had an increased capacity to repair DNA damage caused by ionising radiation, reduce ROS production, and were less susceptible to apoptosis than parental cells, indicating that sarcospheres had highly activated basal DNA repair mechanisms (51).

Meanwhile, another study demonstrated that CSCs could be isolated from MNNG/HOS (Human osteosarcoma) as they can form sarcospheres when cultured in poly-HEMA-coated plates supplemented with growth factor (52). These stem-like cells form floating spherical colonies known as sarcospheres or CSCs when cultured in serum-free medium supplemented with growth factors under anchorage-independent conditions. They retain their ability to form new colonies for at least four subsequent generations, demonstrating their self-renewal ability, which is a hallmark of stem-like cells. Protein expression of pluripotency-related transcription factors OCT4 and NANOG were significantly upregulated in the first and fourth-generation spheres compared to corresponding parental. This provides evidence for the stem-like nature and maintenance over time.

In another study conducted by Paiva-Oliveira et al. (53) also demonstrated that stem-like cells were detected in human osteosarcoma cell lines MNNG/HOS and MG-63 when cultures on poly-HEMA-coated plates supplemented with serum-free medium. Adherent cells grew in suspension and formed compact spherical colonies, termed spheres. These cells were found to have the ability to generate new spherical colonies in subsequent generations, indicating their self-renewal capacity.

The stemness properties of these cells were confirmed by the expression of pluripotency-related transcription factors OCT4, NANOG, and SOX2, which are crucial for maintaining self-renewal and pluripotency in embryonic stem cells (ESCs). The mRNA expression analysis showed that SOX2 expression was increased by 5.5- and 20-fold in spheres relative to the parental cell lines MNNG/HOS and MG-63. Additionally, sphere-forming cells showed a variable expression pattern of drug resistance-related genes, including the isoforms ALDH1A1, ALDH2, and ALDH7A1 of the aldehyde dehydrogenase (ALDH) and the ATP-binding cassette transporters ABCB1 and ABCG2, which are recognised as stem-like markers.

Scaffold-free 3D culture

Scaffold-free 3D culture prevents cells from adhering to the surface of the cell culture container by applying a coating of non-adherent materials. The lack of adherent surfaces mediates cell-to-cell adhesion and the formation of spheroids (54). The liquid overlay and hanging drop technique is a liquid-based scaffold-free approach that produces spheroids using mono- or multicellular (co-cultures) methods with the help of gravity (55-57). Liquid overlay is the most used technique in isolating and enriching cancer stem cells in osteosarcoma by preventing cell adherence using ultra-low attachment binding plates (21). Meanwhile, hanging drop plates uses the force of gravity to form spheroids through self-aggregation. Due to their reproducibility, the hanging drop plate technique can be utilised for various purposes, such as investigating cell organisation, tissue formation, tumour biology, and embryonic development (54).

Low and ultralow binding plate

Low and ultralow binding plate is a 3D culture that uses plate coated with an inert substance such as agarose. This type of culture is useful to isolate cancer stem cells or tumor-initiating cells as the substance is useful to minimise cell attachment with the use of serum-free culture medium.

Study by Guo et al. (58) used ultralow attachment plates to investigate the role of miR-335 in osteosarcoma stem cells with regard to their stem-like properties. The CSCs formed spheres when cultured in serum-free conditions. Spheroids from all three osteosarcoma cell lines MG-63, 143B, and U2OS showed a significant decrease in the expression of miR-335 mRNA. The ability of spheroid formation is essential to evaluate the self-renewal capacity of stem cell-like cells in vitro. The findings compared the ability of high miR-335 and low miR-335 expression cells to generate spheroids. The expression levels of miR-335 mRNA can be used to determine the stem cell-like features in osteosarcoma cell lines. The results indicated that low miR-335-expressing cells produced more spheroids than high miR-335-expressing cells. The findings suggest that miR-335 expression is inversely associated with stem cell-like characteristics in osteosarcoma.

On the other hand, a study conducted by Honoki et al. (15) aimed to correlate the expression of aldehyde dehydrogenase 1 (ALDH1) as a stem cell marker for identifying cancer stem cells in osteosarcoma MG-63 and HT1080 fibrosarcoma cell lines. ALDH1 positive and negative cell populations were isolated from MG-63 cell line and cultured into an ultra-low attachment using a cell sorter. The findings showed cell population capable of forming spheroids in serum-free and anchorage-independent conditions demonstrated increased expression of ALDH1 mRNA and stem cell-related genes, including OCT3/4, NANOG, SOX2, and Stat3. Sarcospheres derived from the MG63 cell line are highly resistant to chemotherapy with doxorubicin and cisplatin compared to adherent cells grown in a monolayer. Based on these findings, sphere-forming cells with elevated levels of ALDH1 may represent sarcoma stem cells with strong chemoresistance properties.

Meanwhile, a study by Fujii et al. (11) aimed to demonstrate whether the human osteosarcoma cell lines Ewing's sarcoma HTB166, MG-63, and fibrosarcoma HT1080 grown in ultra-low attachment plate possess stem-like properties. The results demonstrated that sarcospheres we formed in all cell lines under anchorage-independent and serum-starved conditions. These sarcospheres demonstrated stem-like characteristics, including self-renewal ability and elevated expression of stem cell-related genes OCT3/4, NANOG, SOX2 and DNA repair enzyme genes MLH1 and MSH2. The sarcospheres were highly resistant to drugs such as doxorubicin and cisplatin. The addition of caffeine, a DNA repair inhibitor, improved the efficacy of these drugs. These findings suggest that Ewing's sarcoma HTB166, MG-63, and fibrosarcoma HT1080 cell lines possess populations of stem-like cells that exhibit potent drug resistance and that the efficacy of chemo drugs against sarcomas may be enhanced by DNA repair inhibitors.

Hanging drop

The hanging drop technique remains the most used approach for producing spherical micro-masses from immortalised and primary cell lines. In this method, cells are suspended in a small droplet of medium, which is hung from the lid of a petri dish or a specialised hanging drop plate. The droplet is flipped upside down, allowing the cells to settle and form a compact spheroid in the hanging droplet. Spherical micro-masses can also be created by co-culturing multiple cell types without using synthetic materials as support, relying instead on gravity (59).

A study by Gatti et al. (60) aimed to form multicellular tumour spheroids from canine osteosarcoma cell line OSA1, OSA2, and OSA3 using the hanging drop technique adapted from Berens et al. (61) to study the effect of metformin on tumour spheroid invasion and stemness. The spheroids were grown under serum-free medium culture conditions. The untreated OSA1 and OSA2 CSC spheroids demonstrated the capacity to self-renew, producing new sarcospheres with the same efficiency, even after six passages. However, the ability of OSA1 CSCs to self-renew was significantly reduced after treatment with metformin, as they could not form new spheroids after two passages. In OSA2 CSCs, the ability to self-renew was also progressively reduced by metformin treatment over multiple passages.

Table1: 3D culture scaffolds for osteosarcoma CSCs

Material	Method	Cell line	Drug screening	Stemness markers	Refs
Scaffold-based Natural polymers	Collagen type 1	Human osteosarcoma MG-63	Plasma Activated Ringer's (PAR)	SOX2, OCT3/4, NANOG	(35)
		Human osteosarcoma MG-63, SAOS-2	-	OCT4, NANOG, SOX-2, NOTCH-1, HIF-1α	(36)
	Agar	SAOS-2, CD133$^+$, CD133$^-$, SAOS-2	-	Sox2, OCT3/4, Nanog, Nestin, CD133, Ki-67	(29)
	Matrigel	MG-63, 3AB-OS osteosarcoma CSCs		CD133, OCT3/4, SOX2, NANOG, HMGA2, h-TERT, Nucleostemin, Nestin, Nucleostemin, ABCG2, Lin28B, Ki-67, PCNA	(37)

Table1: 3D culture scaffolds for osteosarcoma CSCs (continued)

Material	Method	Cell line	Drug screening	Stemness markers	Refs
	Alginate	Osteosarcoma specimen from patient	Epirubicin	OCT3/4, NANOG	(40)
	Bacterial cellulose	SaOS-2, CD133$^+$, CD133$^-$,	-	CD133, OCT4 and VEGF	(42)
	Hyaluronic acid	HOS, U2-OS, 143B	-	-	(44)
Synthetic polymers	Polyethylene glycol diacrylate (PEGDA) hydrogel	U2OS	-	CD44, EGFR, and ABCG2	(49)
	Poly-HEMA	human MNNG/HOS	Cisplatin, Doxorubicin, Methotrexate, Erastin, Apatinib, Sorafenib, Everolimus, Chloroquine	CD73, CD90, CD103, OCT4, NANOG	(51)
Scaffold-free	Low and ultralow binding plate	MG-63, 143B, and U2OS	-	miR-335	(58)
	Hanging drop	canine osteosarcoma cell line OSA1, OSA2, and OSA3	Metformin, doxorubicin, cisplatin	OCT4, CD117, STAT3, SOX2, CD133	(60)

Conclusion

Cancer stem cell (CSC) biology has garnered significant global attention in recent years. The development of targeted therapies against CSCs holds promise for improving overall survival rates in various types of cancer. For a long time, 2D cell cultures were deemed a reliable method to gain insight into tumour biology. However, numerous studies have indicated that 2D cultures fail to accurately replicate the true nature of tumours, as several treatments that were effective in 2D failed in clinical trials. To address this issue, 3D culture approaches were developed to mimic the tumour microenvironment. The use of natural materials similarly presents in the bone extracellular matrix (ECM) or synthetic materials as scaffolds for generating 3D bone cultures have demonstrated promising outcomes for investigating tumour invasion, metastasis, angiogenesis, and chemotherapy drug development.

Advancements in 3D culture techniques has been useful in studying the behaviour of CSCs in tumours which causes treatment resistance and may contribute to our understanding of mechanism-based therapies for cancer. However, it is important to consider the advantages and limitations of each type of culture scaffold before selecting the optimal approach for a particular experimental question. Culturing osteosarcoma CSCs with scaffold allows controlling the chemical composition, structure, porosity, stiffness, and shape of the 3D matrix, influencing cell-to-cell interactions, proliferation, and migration abilities of tumour cells (62).

The development of innovative technologies in 3D cultures has led to the emergence of 3D bioprinting that allows customable scaffolds. This technology can be combined with other 3D techniques to mimic the native tumour microenvironment of bone tumours, including cell-to-cell, cell adhesion, proliferation, migration, extracellular matrix structure, tissue interactions, and hypoxia. This 3D technological advancement presents a remarkable opportunity for identifying new therapeutic targets and drug discovery in bone sarcoma.

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Competing interests

The authors declare that they have no competing interests

References

1. Harris MA, Hawkins CJ. Recent and ongoing research into metastatic osteosarcoma treatments. International Journal of Molecular Sciences. 2022;23(7):3817.
2. Menendez N, Epelman M, Shao L, Douglas D, Meyers AB, editors. Pediatric osteosarcoma: pearls and pitfalls. Seminars in Ultrasound, CT and MRI; 2022: Elsevier.
3. Abarrategi A, Tornin J, Martinez-Cruzado L, Hamilton A, Martinez-Campos E, Rodrigo JP, et al. Osteosarcoma: cells-of-origin, cancer stem cells, and targeted therapies. Stem cells international. 2016;2016: 3631764
4. Casali PG, Bielack S, Abecassis N, Aro H, Bauer S, Biagini R, et al. Bone sarcomas: ESMO–PaedCan–EURACAN Clinical Practice Guidelines for diagnosis, treatment and follow-up. Annals of Oncology. 2018;29:iv79-iv95.
5. Moukengue B, Lallier M, Marchandet L, Baud'huin M, Verrecchia F, Ory B, et al. Origin and therapies of osteosarcoma. Cancers. 2022;14(14):3503.
6. Paiva-Oliveira DI, Martins-Neves SR, Abrunhosa AJ, Fontes-Ribeiro C, Gomes CMF. Therapeutic potential of the metabolic modulator Metformin on osteosarcoma cancer stem-like cells. Cancer Chemother Pharmacol. 2018;81(1):49-63.
7. Subramaniam D, Kaushik G, Dandawate P, Anant S. Targeting Cancer Stem Cells for Chemoprevention of Pancreatic Cancer. Current Medicinal Chemistry. 2018;25(22):2585-94.
8. Nouri M, Caradec J, Lubik AA, Li N, Hollier BG, Takhar M, et al. Therapy-induced developmental reprogramming of prostate cancer cells and acquired therapy resistance. Oncotarget. 2017;8(12):18949.
9. Gibbs CP, Kukekov VG, Reith JD, Tchigrinova O, Suslov ON, Scott EW, et al. Stem-like cells in bone sarcomas: implications for tumorigenesis. Neoplasia. 2005;7(11):967-76.
10. Siclari VA, Qin L. Targeting the osteosarcoma cancer stem cell. Journal of orthopaedic surgery and research. 2010;5:1-10.
11. Fujii H, Honoki K, Tsujiuchi T, Kido A, Yoshitani K, Takakura Y. Sphere-forming stem-like cell populations with drug resistance in human sarcoma cell lines. International journal of oncology. 2009;34(5):1381-6.
12. Wang L, Park P, Lin C-Y. Characterization of stem cell attributes in human osteosarcoma cell lines. Cancer biology & therapy. 2009;8(6):543-52.
13. Rodrigues MFSD, Xavier FCdA, Andrade NP, Lopes C, Miguita Luiz L, Sedassari BT, et al. Prognostic implications of CD44, NANOG, OCT4, and BMI1 expression in tongue squamous cell carcinoma. Head & neck. 2018;40(8):1759-73.
14. Kozovska Z, Patsalias A, Bajzik V, Durinikova E, Demkova L, Jargasova S, et al. ALDH1A inhibition sensitizes colon cancer cells to chemotherapy. BMC cancer. 2018;18(1):1-11.
15. Honoki K, Fujii H, Kubo A, Kido A, Mori T, Tanaka Y, et al. Possible involvement of stem-like populations with elevated ALDH1 in sarcomas for chemotherapeutic drug resistance. Oncology reports. 2010;24(2):501-5.
16. Sun DX, Liao GJ, Liu KG, Jian H. Endosialin-expressing bone sarcoma stem-like cells are highly tumor-initiating and invasive. Molecular Medicine Reports. 2015;12(4):5665-70.
17. Menéndez ST, Gallego B, Murillo D, Rodríguez A, Rodríguez R. Cancer stem cells as a source of drug resistance in bone sarcomas. Journal of Clinical Medicine. 2021;10(12):2621.
18. Vallette FM, Olivier C, Lezot F, Oliver L, Cochonneau D, Lalier L, et al. Dormant, quiescent, tolerant and persister cells: Four synonyms for the same target in cancer. Biochemical pharmacology. 2019;162:169-76.
19. Bassi G, Panseri S, Dozio SM, Sandri M, Campodoni E, Dapporto M, et al. Scaffold-based 3D cellular models mimicking the heterogeneity of osteosarcoma stem cell niche. Scientific Reports. 2020;10(1):22294.
20. Alghuwainem A, Alshareeda AT, Alsowayan B. Scaffold-free 3-D cell sheet technique bridges the gap between 2-D cell culture and animal models. International journal of molecular sciences. 2019;20(19):4926.
21. Hickman JA, Graeser R, de Hoogt R, Vidic S, Brito C, Gutekunst M, et al. Three-dimensional models of cancer for pharmacology and cancer cell biology: capturing tumor complexity in vitro/ex vivo. Biotechnology journal. 2014;9(9):1115-28.
22. Petersen OW, Rønnov-Jessen L, Howlett AR, Bissell MJ. Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells. Proceedings of the National Academy of Sciences. 1992;89(19):9064-8.
23. Kilian KA, Bugarija B, Lahn BT, Mrksich M. Geometric cues for directing the differentiation of mesenchymal stem cells. Proceedings of the National Academy of Sciences. 2010;107(11):4872-7.
24. Anderson NM, Simon MC. The tumor microenvironment. Current Biology. 2020;30 (16):R921-R5.
25. Edmondson R, Broglie JJ, Adcock AF, Yang L. Three-Dimensional Cell Culture Systems and Their Applications in Drug Discovery and Cell-Based Biosensors. ASSAY and Drug Development Technologies. 2014;12(4):207-18.
26. Horvath P, Aulner N, Bickle M, Davies AM, Nery ED, Ebner D, et al. Screening out irrelevant cell-based models of disease. Nature Reviews Drug Discovery. 2016;15(11):751-69.
27. Ravi M, Ramesh A, Pattabhi A. Contributions of 3D cell cultures for cancer research. Journal of cellular physiology. 2017;232(10):2679-97.
28. Villasante A, Vunjak-Novakovic G. Tissue-engineered models of human tumors for cancer research. Expert Opinion on Drug Discovery. 2015;10(3):257-68.

29. Ozturk S, Gorgun C, Gokalp S, Vatansever S, Sendemir A. Development and characterization of cancer stem cell-based tumoroids as an osteosarcoma model. Biotechnology and bioengineering. 2020;117(8):2527-39.
30. Jensen C, Teng Y. Is it time to start transitioning from 2D to 3D cell culture? Frontiers in molecular biosciences. 2020;7:33.
31. Hamburger AW, Salmon SE. Primary bioassay of human tumor stem cells. Science. 1977;197(4302):461-3.
32. Kapałczyńska M, Kolenda T, Przybyła W, Zajączkowska M, Teresiak A, Filas V, et al. 2D and 3D cell cultures–a comparison of different types of cancer cell cultures. Archives of Medical Science. 2018;14(4):910-9.
33. Marchini A, Gelain F. Synthetic scaffolds for 3D cell cultures and organoids: applications in regenerative medicine. Critical reviews in biotechnology. 2022;42(3):468-86.
34. Langhans SA. Three-dimensional in vitro cell culture models in drug discovery and drug repositioning. Frontiers in pharmacology. 2018;9:6.
35. Tornín J, Villasante A, Solé-Martí X, Ginebra M-P, Canal C. Osteosarcoma tissue-engineered model challenges oxidative stress therapy revealing promoted cancer stem cell properties. Free Radical Biology and Medicine. 2021;164:107-18.
36. Bassi G, Panseri S, Dozio SM, Sandri M, Campodoni E, Dapporto M, et al. Scaffold-based 3D cellular models mimicking the heterogeneity of osteosarcoma stem cell niche. Scientific Reports. 2020;10(1):1-12.
37. Di Fiore R, Guercio A, Puleio R, Di Marco P, Drago-Ferrante R, D'Anneo A, et al. Modeling human osteosarcoma in mice through 3AB-OS cancer stem cell xenografts. Journal of Cellular Biochemistry. 2012;113(11):3380-92.
38. Andersen T, Auk-Emblem P, Dornish M. 3D cell culture in alginate hydrogels. Microarrays. 2015;4(2):133-61.
39. Hurtado A, Aljabali AA, Mishra V, Tambuwala MM, Serrano-Aroca Á. Alginate: Enhancement strategies for advanced applications. International Journal of Molecular Sciences. 2022;23(9):4486.
40. Zhou S, Li F, Xiao J, Xiong W, Fang Z, Chen W, et al. Isolation and identification of cancer stem cells from human osteosarcom by serum-free three-dimensional culture combined with anticancer drugs. Journal of Huazhong University of Science and Technology [Medical Sciences]. 2010;30:81-4.
41. Saranya RJ, Vani C, Gobikrishnan S. Production and characterization of bacterial cellulose scaffold from Acetobacter sp. for tissue engineering. Journal of Applied Biology and Biotechnology. 2022;10(6):87-94.
42. Gorgun C, Ozturk S, Gokalp S, Vatansever S, Gurhan SID, Urkmez AS. Synergistic role of three dimensional niche and hypoxia on conservation of cancer stem cell phenotype. International journal of biological macromolecules. 2016;90:20-6.
43. Chircov C, Grumezescu AM, Bejenaru LE. Hyaluronic acid-based scaffolds for tissue engineering. Rom J Morphol Embryol. 2018;59(1):71-6.
44. Lin Y, Yang Y, Yuan K, Yang S, Zhang S, Li H, et al. Multi-omics analysis based on 3D-bioprinted models innovates therapeutic target discovery of osteosarcoma. Bioactive Materials. 2022;18:459-70.
45. Liu Z, Vunjak-Novakovic G. Modeling tumor microenvironments using custom-designed biomaterial scaffolds. Current opinion in chemical engineering. 2016;11:94-105.
46. Rijal G, Li W. 3D scaffolds in breast cancer research. Biomaterials. 2016;81:135-56.
47. Ferreira LP, Gaspar VM, Mano JF. Decellularized extracellular matrix for bioengineering physiomimetic 3D in vitro tumor models. Trends in Biotechnology. 2020;38(12):1397-414.
48. Khalili MH, Afsar A, Zhang R, Wilson S, Dossi E, Goel S, et al. Thermal response of multi-layer UV crosslinked PEGDA hydrogels. Polymer Degradation and Stability. 2022;195:109805.
49. Jabbari E, Sarvestani SK, Daneshian L, Moeinzadeh S. Optimum 3D matrix stiffness for maintenance of cancer stem cells is dependent on tissue origin of cancer cells. PloS one. 2015;10(7):e0132377.
50. Zasońska BA, Brož A, Šlouf M, Hodan J, Petrovský E, Hlídková H, et al. Magnetic superporous poly (2-hydroxyethyl methacrylate) hydrogel scaffolds for bone tissue engineering. Polymers. 2021;13(11):1871.
51. Martins-Neves SR, Lopes ÁO, Do Carmo A, Paiva AA, Simões PC, Abrunhosa AJ, et al. Therapeutic implications of an enriched cancer stem-like cell population in a human osteosarcoma cell line. BMC cancer. 2012;12(1):1-16.
52. Gonçalves C, Martins-Neves SR, Paiva-Oliveira D, Oliveira VE, Fontes-Ribeiro C, Gomes CM. Sensitizing osteosarcoma stem cells to doxorubicin-induced apoptosis through retention of doxorubicin and modulation of apoptotic-related proteins. Life sciences. 2015;130:47-56.
53. Paiva-Oliveira DI, Martins-Neves SR, Abrunhosa AJ, Fontes-Ribeiro C, Gomes CM. Therapeutic potential of the metabolic modulator Metformin on osteosarcoma cancer stem-like cells. Cancer chemotherapy and pharmacology. 2018;81:49-63.
54. Munoz-Garcia J, Jubelin C, Loussouarn A, Goumard M, Griscom L, Renodon-Cornière A, et al. In vitro three-dimensional cell cultures for bone sarcomas. Journal of Bone Oncology. 2021;30:100379.
55. Arai K, Sakamoto R, Kubota D, Kondo T. Proteomic approach toward molecular backgrounds of drug resistance of osteosarcoma cells in spheroid culture system. Proteomics. 2013;13(15):2351-60.
56. Baek N, Seo OW, Kim M, Hulme J, An SSA. Monitoring the effects of doxorubicin on 3D-spheroid tumor cells in real-time. OncoTargets and Therapy. 2016;Volume 9:7207-18.
57. Zanoni M, Piccinini F, Arienti C, Zamagni A, Santi S, Polico R, et al. 3D tumor spheroid models for in vitro therapeutic screening: a systematic approach to enhance the biological relevance of data obtained. Scientific Reports. 2016;6(1):19103.

58. Guo X, Yu L, Zhang Z, Dai G, Gao T, Guo W. miR-335 negatively regulates osteosarcoma stem cell-like properties by targeting POU5F1. Cancer cell international. 2017;17(1):1-9.
59. Shri M, Agrawal H, Rani P, Singh D, Onteru SK. Hanging drop, a best three-dimensional (3D) culture method for primary buffalo and sheep hepatocytes. Scientific Reports. 2017;7(1):1203.
60. Gatti M, Solari A, Pattarozzi A, Campanella C, Thellung S, Maniscalco L, et al. In vitro and in vivo characterization of stem-like cells from canine osteosarcoma and assessment of drug sensitivity. Experimental cell research. 2018;363(1):48-64.
61. Berens EB, Holy JM, Riegel AT, Wellstein A. A cancer cell spheroid assay to assess invasion in a 3D setting. JoVE (Journal of Visualized Experiments). 2015(105):e53409.
62. Liu C, Liu Y, Xu X-x, Wu H, Xie H-g, Chen L, et al. Potential effect of matrix stiffness on the enrichment of tumor initiating cells under three-dimensional culture conditions. Experimental cell research. 2015;330(1):123-34.