

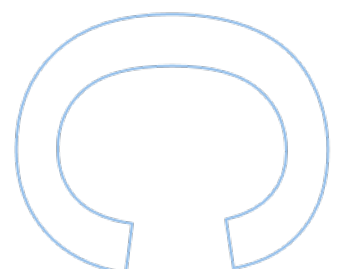
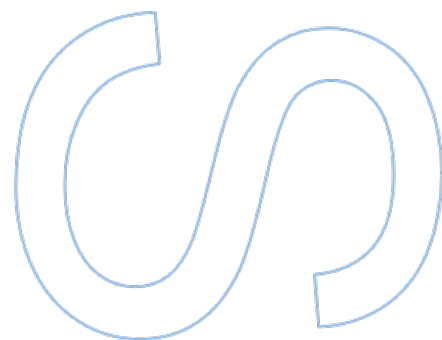
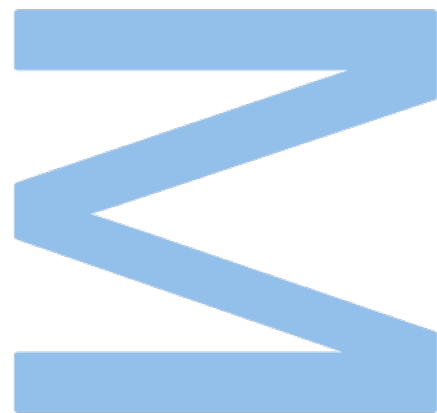
Development and characterization of personalized magnetic spheroids as advanced 3D tumor models

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2024/2025



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Dissertation carried out as part of the Master in Biochemistry
2024/2025

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BIOMEDICAL SCIENCES**



Dedicated to myself and my family

Acknowledgements

Completing this thesis has been a remarkable challenge, not only academically but also personally. I would like to express my deep gratitude to all those who have contributed in some way to this journey.

Firstly, I would like to thank my supervisor, Professor Manuela Gomes, for her rigorous guidance, constant support and the knowledge she shared throughout the process. Her professionalism and demands were fundamental to the realization of this work.

My special thanks also go to Adriana Vinhas and Mehrzad Zargarzadeh, for all her support and dedication over the months, and to all my lab colleagues, Bárbara Adão, Sandro Caetano, Érica Pereira and Ana Gonçalves, for their availability, spirit of mutual help and collaborative working environment. Last but not least, I would also like to thank Ana Maria for all her support and loaned material.

A special thank you to my family (grandparents, parents, brother, godparents and cousins), who have always supported me unconditionally with words of encouragement in the most challenging moments. A special thank you to my grandmother Rosália, my brother and my parents for all the travelling and endless waiting times. To my friends, thank you for your company, patience and for reminding me to breathe on the most intense days.

Finally, I would like to thank all the people who, directly or indirectly, helped make this project possible.

To everyone, my sincere thanks.

Resumo

O osteossarcoma (OS) é um tipo de cancro extremamente raro, para o qual as opções de tratamento são muito limitadas devido à sua heterogeneidade, resistência aos fármacos e elevada ocorrência de metástases. Para o desenvolvimento de terapias mais eficientes, é útil desenvolver modelos que mimetizem mais fielmente o microambiente tumoral *in vivo*, a fim de compreender melhor a doença e os mecanismos terapêuticos.

Assim, o principal objetivo deste estudo foi desenvolver esferóides magnéticos 3D de OS através da incorporação de partículas magnéticas. A estimulação subsequente por campos magnéticos externos pode permitir orientar a deposição assimétrica de ECM e, assim, gerar forças mecânicas localizadas dentro dos esferóides, imitando o endurecimento da ECM tumoral que dificulta a penetração de fármacos quimioterápicos.

Para este efeito, foram desenvolvidos e otimizados esferóides magnéticos a partir de uma mistura de MSCs e células Saos-2 numa proporção de 1:3. Os esferóides foram então estimulados utilizando um biorreator magnético e depois incorporados numa matriz 3D para estudar a invasividade e a penetração de fármacos (doxorrubicina).

A partir dos resultados obtidos, foi possível desenvolver esferóides consistentes e viáveis incorporando partículas magnéticas. Para além disso, a aplicação de um campo magnético mostrou estimular a deposição de matriz extracelular. A incorporação dos esferóides numa matriz mostrou que a estimulação gera maior invasão e proliferação. Finalmente, os estudos de penetração de fármacos mostraram que os esferóides estimulados têm uma maior diminuição da viabilidade.

Em conclusão, os resultados obtidos evidenciam que os modelos 3D desenvolvidos podem imitar aspetos específicos da rigidez tumoral, invasividade e resistência a fármacos, que com refinamentos adequados podem tornar-se ferramentas cruciais para estudar a remodelação tumoral ou modular a MEC, com o objetivo de melhorar a eficácia de fármacos/tratamentos antitumorais.

Palavras-chave: Biorreator; Campo magnético; Invasão celular; Matriz extracelular; Microambiente tumoral; MSCs; NanoShuttle; Partículas magnéticas; Penetração de fármacos; Rigidez tumoral; Saos-2; Terapias antitumorais.

Abstract

Osteosarcoma (OS) is an extremely rare type of cancer, for which the treatment options are very limited due to its heterogeneity, drug resistance and high occurrence of metastases. For the development of more efficient therapies, it is necessary to develop models that mimic the *in vivo* tumor microenvironment more faithfully in order to understand better the disease and therapeutic mechanisms.

Therefore, the main aim of this study was to develop 3D magnetic OS spheroids through the incorporation of magnetic particles. The subsequent stimulation by external magnetic fields may enable to guide asymmetric ECM deposition and thus generate localized mechanical forces within spheroids mimicking the stiffening of tumor ECM that difficulties chemo drug penetration.

For this purpose, it was developed and optimized magnetic spheroids from a mixture of MSCs and Saos-2 cells in a 1:3 ratio. The spheroids were then stimulated using a magnetic bioreactor and then incorporated into a 3D matrix to study invasiveness and drug (doxorubicin) penetration.

From the results obtained, it was possible to develop consistent and viable spheroids incorporating magnetic particles. Furthermore, the application of a magnetic field showed to stimulate the deposition of extracellular matrix. Incorporating the spheroids into a matrix showed that stimulation generates greater invasion and proliferation. Finally, drug penetration studies shown that stimulated spheroids have a biggest decrease in viability.

In conclusion, the results obtained evidence that the developed 3D models might mimic specific aspects of tumor stiffness, invasiveness and drug resistance, that with appropriate refinements may become crucial tools for studying the tumor remodeling or modulating the ECM, with the aim of improving the efficacy of antitumor drugs/treatments.

Keywords: Anti-tumor therapies; Bioreactor; Cell invasion; Drug penetration; Extracellular matrix; Magnetic field; Magnetic particles; MSCs; NanoShuttle; Saos-2; Tumor microenvironment; Tumor stiffness.

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List of Abbreviations

2D – Two-dimensional culture
3D – Three-dimensional culture
AJCC – American Joint Committee on Cancer
CDK4 – Cyclin-Dependent Kinase 4
CSCs – Osteosarcoma Cancer Stem Cells
DOX – Doxorubicin
ECM – Extracellular Matrix
ESFT – Ewing’s sarcoma family tumors
EWSR1 – Ewing sarcoma RNA-binding protein 1
FBS – Fetal bovine serum
FLI1 – Friend Leukemia Integration 1
hFOB – human fetal osteoblast cells
HIF – Hypoxia Induced Factor
hMSCs – Human Mesenchymal Stem Cells
IGF-1 – Insulin-like growth factor type 1
IL-6 – Interleukin-6
MDM2 – Mouse Double Minute 2 homolog
MMPs – Matrix Metalloproteinases
MSCs – Mesenchymal Stem Cells
ON – Over-night
OS – Osteosarcoma
PNET – Peripheral primitive neuroectodermal tumors
PTHrP – Parathyroid hormone-related protein
RANKL – Receptor Activator of Nuclear Factor-kB Ligand
RB – Retinoblastoma
RB1 - Retinoblastoma protein
RT – Room temperature
SD – Standard deviation
S phase – Synthesis phase
TGF- β - Transforming growth factor-beta
VEGF – Vascular Endothelial Growth Factor

1. Introduction

1.1. Motivation and Thesis Aims

Osteosarcoma (OS) is an extremely heterogeneous and rare type of cancer that occurs predominantly in children and young adults. Treatment options for this type of tumor are quite limited due to their heterogeneity, resistance to drugs and the high occurrence of metastases [1], [2]. The extracellular matrix (ECM) plays a central role in the biology of OS, modulating not only the tumor microenvironment, but also processes such as growth, invasion, angiogenesis and metastasis. In the tumor context, the ECM is not a static structure - it is actively remodeled by tumor and stromal cells, directly influencing the aggressiveness of OS. OS cells express enzymes such as matrix metalloproteinases (MMPs), particularly MMP-2, MMP-9 and MMP-13, which degrade ECM components such as collagen and fibronectin. This degradation facilitates cell migration, paving the way for tumor invasion, and releases growth factors linked to the MEC, such as TGF- β , VEGF or FGF, which intensify malignant behavior [3].

Studying ECM modulation in OS is crucial because it directly affects the invasive and metastatic capacity of the tumor, especially to the lung, allows the identification of tumor progression biomarkers and potential therapeutic targets, such as MMPs or integrins, and can improve 3D tumor modelling, better reflecting tumor physiology compared to two-dimensional (2D) cultures. Modulation of ECM in OS is closely linked to several signaling pathways, such as PI3K/AKT/mTOR, which promotes cell survival and motility in response to cell-MEC interaction; MAPK/ERK, which is activated by integrins and growth factors, regulates proliferation and invasiveness; TGF- β /SMAD, which modulates expression of MMPs and reorganization of ECM, as well as promoting EMT-like features; FAK/Src: mechano-sensitive pathway linking ECM alterations to cytoskeleton reorganization and cell migration [4], [5], [6].

Currently, 2D cell cultures are the predominant *in vitro* testing systems used for drug screening [7]. However, this approach inadequately represents tumor heterogeneity, three-dimensional (3D) architectural structures, and physiological barriers, resulting in inaccurate pharmacological assessments. In contrast, biomimetic 3D *in vitro* tumor models are emerging as effective tools for the more precise evaluation of drug toxicity [7], [8]. 3D models can replicate specific characteristics of the tumor microenvironment, thereby providing valuable insights into the intratumoral penetration and diffusion dynamics of drugs. Despite the advantages of 3D models, *in vivo* validation of treatments

remains vital during the preclinical phase. While an array of advanced *in vivo* tumor models is available such as genetically engineered mice and patient-derived xenografts, such methodologies also present significant limitations. In fact, *in vivo* studies can be prohibitively expensive, time-consuming, and subject to ethical considerations, which may limit the scope of experimentation [9, p. 3]. Additionally, the biological discrepancies between animal models and human physiology can occasionally compromise the accuracy of clinical outcome predictions [10], [11].

Spheroids are recognized as a key and versatile scaffold-free method for promoting 3D cellular growth in tumor biology models. These structures are formed by aggregates of cells that spontaneously organize into multicellular configurations, effectively replicating essential features of the *in vivo* tumor microenvironment, an aspect that traditional 2D cell cultures lacks [9], [12].

By employing high-throughput spheroid culture techniques, researchers can significantly enhance the effectiveness of their experimental designs allowing for the simultaneous evaluation of the effects of multiple compounds on cellular behavior, which is especially beneficial in drug research and development. It facilitates rapid assessment of therapeutic efficacy and toxicity in an environment that more closely resembles actual physiological conditions [13].

Additionally, the flexibility of spheroid cultures allows for adjustments in size, composition, and cellular density, enabling the creation of tailored models that address specific research challenges or therapeutic strategies. This adaptability makes spheroids invaluable for exploring various aspects of tumor biology, including mechanisms of drug resistance, metastatic potential, and the interactions within the tumor microenvironment. Ultimately, tumor spheroids offer a robust platform that enhances our understanding of cancer biology and supports the advancement of therapeutic interventions [14].

The primary objective of this thesis is to develop magnetic OS spheroids that integrate magnetic particles within the OS cells (Saos-2). This system enables the stimulation of ECM modification using external magnetic fields. This unique technique aims to elucidate the connection between the mechanical properties of the ECM and tumor biology, hence facilitating the development of more effective treatment regimens [15].

1.2. Specific Objectives

This thesis presents the development of a 3D *in vitro* OS model that emphasizes the incorporation of magnetic particles (Nanoshuttle-PL particle) into the spheroids. This configuration is expected to allow to replicate alterations in the ECM of cells when subjected to an external magnetic field that recapitulate changes occurring in tumor ECM that can influence drug penetration, for example.

The most important question this thesis seeks to answer is “Does the combination of spheroids with magnetic particles improve the *in vitro* representation of the OS tumor microenvironment in terms of ECM modulation?” In particular, do these magnetic particles influence how OS spheroids respond to the anticancer drug (Doxorubicin).

Therefore, the first step of this work was to develop optimized OS spheroids, using SaOS-2 cells, that incorporated Nanoshuttle particles. This process involved a series of optimizations, including testing various methods and figuring out the best cell density for spheroid formation in terms of cells viability and structural integrity of spheroids. Once these spheroids were made, we focused on evaluate the spheroid's ECM in terms of its stiffness in the presence of external magnetic field. At this stage, several biological assays were performed to cells viability and proliferation over time as well as Phalloidin staining. To replicate the natural OS tumor enhancing essential cell-ECM interactions vital for tumor proliferation, the subsequent steps involved encapsulating produced magnetic spheroids with and without the application of a magnetic field within a hydrogel matrix (Matrigel) [16]. This evaluation was involved conducting viability and proliferation assays, immunofluorescent staining, as well as invasion studies.

The ability of drugs to penetrate tumors is crucial since the effectiveness of most therapies depends on their capacity to reach the tumor cells. When a drug is administered, it must be able to overcome the tumor's physical barrier to access the malignant cells and deliver its therapeutic action effectively. [17], [18]. Furthermore, if drug penetration is inadequate, tumor cells may develop resistance to the treatment. This resistance occurs because only a portion of the cells is affected by the drug, causing the unaffected cells to continue proliferating, leading to treatment failure [19]. Doxorubicin (DOX) dose-response testing is a fundamental aspect add of cancer research and therapeutic development [12]. By evaluating the effects of varying concentrations of DOX on magnetic spheroids, the drug's cytotoxic mechanisms were assessed.

Ultimately, our goal was to investigate the efficacy of anti-cancer drug (DOX) by assessing its penetration depth and distribution within the spheroids subjected to magnetic stimulation, in comparison to those not exposed to a magnetic field [20].

Thus, the proposed methodology is expected to enable the production of 3D tumor models, allowing for the analysis of how ECM modulation in magnetic spheroids influences cell invasion and drug penetrability. Finally, it is anticipated that this work will provide insights into the influence of tumor progression under magnetic stimulation.

In summary, this study aims to establish the potential of the proposed method for using these cancer models to study new therapies and drugs.

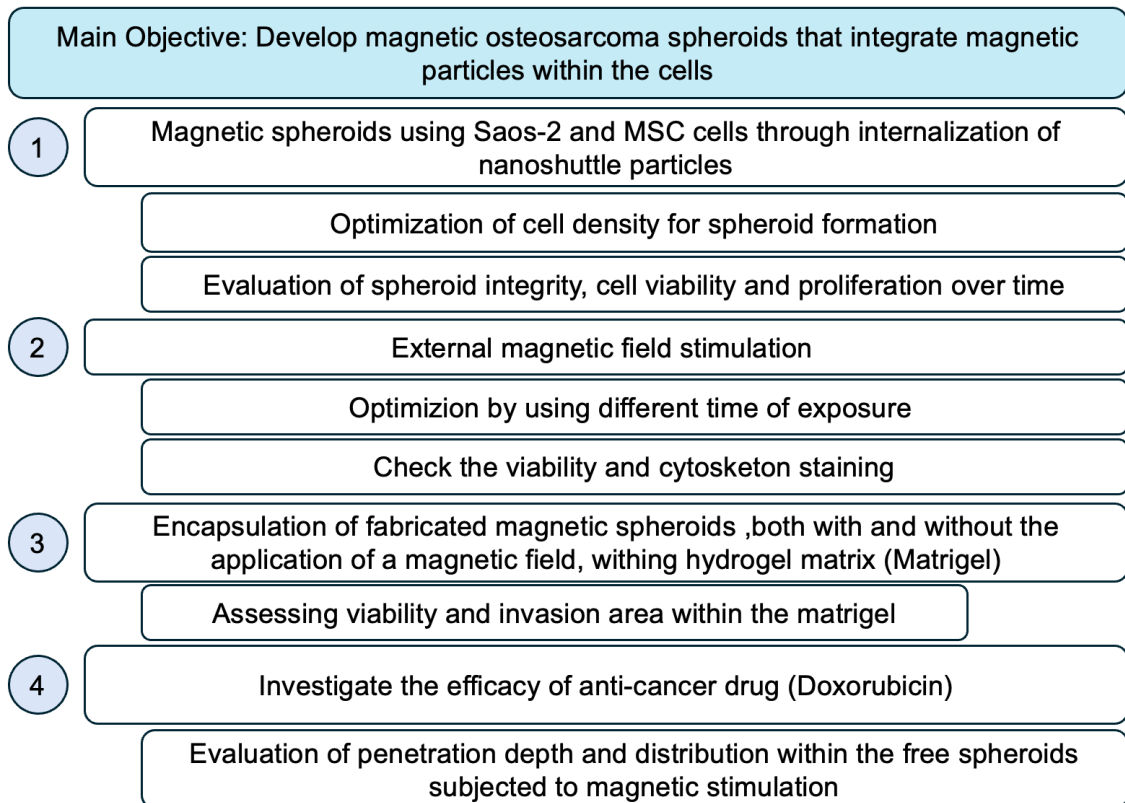


Fig. 1 - Schematical representation of the thesis' objectives.

2.Theoretical overview

2.1. Cancer

Cancer consists of the uncontrolled division of cells, resulting in a tumor, which can be benign or malignant [9]. Nowadays, cancer is one of the leading causes of death in the world, having caused approximately 10 million deaths in 2020 [21]. Tumors arise from the accumulation of mutations in certain genes, such as TP53, retinoblastoma protein (RB1), Mouse Double Minute 2 homolog (MDM2) and Cyclin-Dependent Kinase 4 (CDK4). These genes control cell function as well as cell proliferation and when they are mutated, the cells divide uncontrollably, forming a tumor [22]. The TME includes both tumor and stromal cells, along with a variety of ECM components [9]. In this 3D setting, how these cells function and behave is shaped by their interactions with one another, their interactions with the ECM, and by factors such as gradients, growth factors, secreted factors, and oxygen availability [23].

The differentiation of high-grade and low-grade tumors is of utmost importance in the case of malignant tumors, to anticipate local recurrence and potential metastases [24]. In 90% of cancer cases, mortality and morbidity are caused by metastases, while only 10% are due to the primary tumor. [25]. When the tumor is confined to a single site and does not spread to other organs, it is considered benign. However, if it invades to surrounding tissues and organs through the blood or lymph, the tumor is considered malignant [26]. Tumors develop when cells gain the ability to penetrate and destroy healthy tissue while growing at an uncontrolled rate. In this way, through metastasis, cancer spreads throughout the body [9].

2.2. Musculoskeletal cancer: Current treatments and Challenges

Musculoskeletal cancer develops when there is uncontrolled cell division, forming an abnormal mass in the skeletal muscle system [26]. The presence of a mass in the musculoskeletal system is always a cause for clinical concern, since distinguishing between benign and malignant tumors remains a significant diagnostic challenge. Musculoskeletal tumors can occur in any age group, although their prevalence is more significant in children and adolescents [27], [28]. It is estimated that more than 50% of

patients diagnosed with bone tumors are under 30 years of age, which highlights the importance of this age group in the epidemiology of these neoplasms [27].

Malignant neoplasms of the musculoskeletal system can be classified as primary, when they originate directly from mesenchymal tissues such as bone, cartilage or skeletal muscle, or secondary, when they result from the metastatic spread of primary tumors located in other organs [27], [29]. Despite their clinical relevance, malignant musculoskeletal tumors are rare, accounting for less than 1% of all malignant neoplasms diagnosed. Furthermore, they are a heterogeneous group, both histologically and biologically, which makes early diagnosis and appropriate treatment difficult [27], [30].

Musculoskeletal tumors originate in the musculoskeletal system, that is, in the bones, muscles, tendons, fat, blood vessels, cartilage, and other supportive tissues [28],[31]. This type of tumor is divided into bone tumors and soft tissue tumors. Bone tumors include tumors such as OS and chondrosarcoma. Soft tissue tumors, which consist of supportive tissues, originate in muscles, adipose tissue, connective tissue, and other soft tissues [28]. In the pediatric paradigm, 5% of cancers are bone sarcomas, and 15% of cancers are soft tissue sarcomas [32].

2.3. Different types of musculoskeletal cancers

Musculoskeletal tumors are indeed one of the most challenging areas in diagnostic pathology. According to the 8th edition of the American Joint Committee on Cancer (AJCC) staging system, based on the TNM Classification of Malignant Tumors for bone and soft tissue sarcomas. In the acronym: T indicates the dimensions of the primary tumor and assesses whether it has spread to surrounding tissues, N indicates which nearby lymph nodes are affected, and M indicates the presence of distant metastasis [29],[33]. Bone tumors can be divided into three types based on their location and origin: appendicular skeleton, trunk, skull and facial bones; spine; and pelvis [34], [29]. Soft tissue tumors are divided, based on location, into trunk and extremities, retroperitoneum, head and neck, and visceral sites [29].

There are several types of benign bone tumors including osteochondroma, osteoma, osteoid osteoma, osteblastoma, giant cell tumor, aneurysmal bone cyst, fibrous dysplasia, and enchondroma [35]. The most commonly occurring malignant tumors include chondrosarcoma, OS, Ewing's sarcoma, and synovial sarcoma (Fig. 1) [36].

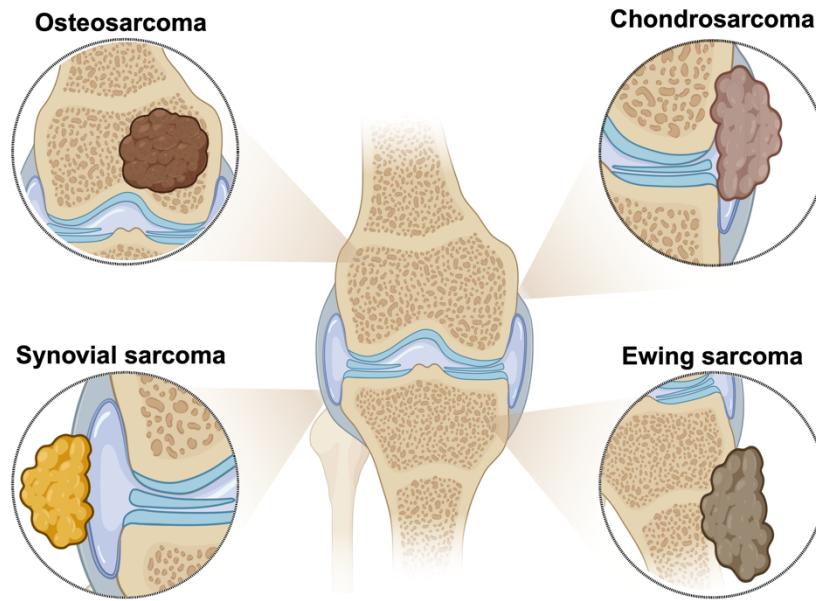


Fig. 2 - Schematic representation of different types of malignant musculoskeletal tumors: OS, Chondrosarcoma, Synovial sarcoma and Ewing sarcoma. Created with BioRender.com

2.3.1. Osteosarcoma

OS is the most common primary bone tumor, highly aggressive and more frequent among children, adolescents, and young adults [20], [37]. This tumor is likely influenced by certain genetic factors related to bone formation diseases. Due to its rarity and the absence of good markers, screening and surveillance for this tumor have not yet been implemented [2]. Comparing carcinomas with sarcomas, carcinomas are the most common malignant tumors and develop in epithelial tissues, while sarcomas develop in connective tissues such as bones and soft tissues [38].

OS originates in osteoblasts of various sizes, called pleomorphic, which produce a large amount of osteoid tissue [39]. This cancer forms in the metaphysis of long bones, which is characterized by intense cell division [2]. This type of cancer, in addition to be highly heterogeneous, has a high malignancy rate due to local aggressiveness and the rapid development of lung metastases [37], [38], [40]. Each year, approximately 3.4 million new cases of this cancer variant are diagnosed worldwide [20]. OS is among the types of tumors with the most severe complications, as they require very aggressive surgical resections, involving the removal of large volumes of tissue, which may subsequently require replacement with prostheses [2], [24].

OS commonly arises in the metaphyseal areas of long bones characterized by rapid growth, notably the distal femur, proximal tibia and humerus [20], [41]. The metaphysis is the part of the bone that contains the growth cartilage and therefore has a greater number of proliferative cells, thus increasing the proliferation rate of the tumor [42].

In the metaphysis, the balance between the activities of osteoblasts, osteoclasts, and mesenchymal precursors is often disrupted [20]. The treatments available and developed over the past 30 years have not shown improvements in the 5-year survival rate [37]. Several factors hinder the success of these treatments, such as the genomic, transcriptomic, and epigenetic heterogeneous nature of OS, its resistance to chemotherapy, drug toxicity, rapid blood clearance, high recurrence rate, and the development of micrometastases [37], [40].

The development of OS is an event caused by various genetic and epigenetic alterations and signals from the bone microenvironment [43]. This type of tumor usually has mutations in the TP53 and retinoblastoma (RB) tumor suppressor genes, which code for the p53 and RB proteins, respectively. The p53 protein is a nuclear transcription factor which is responsible to prevent the uncontrolled proliferation of cells with severe DNA damage through interrupting the cell cycle when the damage can be repaired, or by initiating apoptosis when the damage is beyond repair [44]. In case of DNA damage, RB protein inhibits gene transcription, which is essential for entering the Synthesis phase (S phase) of the cell cycle [45].

Various cell types have been suggested as the genesis of OS within the mesenchymal-osteogenic lineage, including stromal/mesenchymal cells (MSCs), which are primarily located in bone marrow [43], [46]. The accumulation of mutations in MSCs is then associated with the formation of OS cancer stem cells (CSCs), which have the ability to self-renew, differentiate and drive tumor growth by interacting with the ECM [43], [47], [48]. In addition, CSCs are very resistant to chemotherapy due to their ability to repair DNA damage and remove drugs from their cytoplasm, so they have a major influence on tumor recurrences and metastases [43].

The most common symptoms in OS are pain or discomfort in the affected area, limited joint mobility and the appearance of a palpable mass [49]. The diagnosis of this disease is made through a biopsy, which usually shows a high quantity of spindle-shaped malignant cells, with the presence of pleomorphic nuclei and irregular osteoid production [49]. While 95% of OS cases arise spontaneously, some factors elevate the risk of developing this malignancy, including radiation exposure, rapid bone development

during puberty, and pre-existing or inherited bone disorders [38]. Considering the predominant matrix-producing cell types, OS can be classified as osteoblastic, fibroblastic or chondroblastic [49].

2.3.2. Chondrosarcoma

Chondrosarcoma is the second most common primary bone tumor, originating from cartilage-producing cells, with an incidence of 6.63 cases per one million people per year [24], [50]. This type of tumor has a higher prevalence among males, but it varies depending on the subtype [50]. The diagnosis of this type of tumor includes various histological subtypes, with the conventional subtype being the most prevalent, accounting for approximately 85% of cases, followed by the dedifferentiated, clear cell and mesenchymal subtypes [50].

Conventional chondrosarcoma can arise primarily or secondarily from an underlying benign lesion, such as enchondroma or osteochondroma. In comparison to chondrosarcomas, enchondromas are generally smaller and have a chondroid appearance with punctate calcifications, lacking aggressive signs such as cortical rupture or endosteal erosion[51]. Unlike chondrosarcomas, enchondromas are asymptomatic [24]. Mesenchymal chondrosarcoma, a rare subtype, tends to occur in younger individuals, with the highest incidence observed between the ages of 20 and 30 [50], [51].

One of the symptoms of chondrosarcoma is progressive and insidious bone or joint pain, which is the most common among patients and is of great importance in distinguishing chondrosarcomas from benign cartilage tumors. The pain is associated with tumor growth, with a higher percentage of pain complaints among patients with a higher-grade tumor. The pain is usually dull and painful, and may be intermittent, worsening at night [24].

Chondrosarcomas typically occur in the bony skeleton, although a small portion may arise as a primary mass in the soft tissues. In the appendicular skeleton, this tumor is more prevalent in the long bones of the lower limb, with the proximal femur being the most common site. In long bones, tumors usually originate in the medullary cavity of the metaphysis, except for clear cell chondrosarcoma, which originates in the epiphysis. On

the other hand, chondrosarcomas also have a high prevalence in the pelvis, with about 20% of cases arising from the pelvic bones [50], [51].

Early diagnosis and treatment are crucial in order to reduce the disease burden and the possibility of metastasis, thus improving survival. To this day, there are no known and proven innovative methods for the early detection of chondrosarcoma. However, circulating tumor DNA has emerged as an important biomarker for the diagnosis and detection of disease in various types of tumors [24], [50].

2.3.3. Ewing sarcoma (ES)

Ewing's sarcoma family tumors (ESFT) represent a malignant category of pediatric cancer, encompassing Ewing's sarcoma, peripheral primitive neuroectodermal tumors (PNET) and Askin tumors [52]. This type of tumor is rare, and highly aggressive, and typically occurs in the long bones and pelvis, with the potential to rapidly metastasize to the bone marrow and lungs [1], [53]. These types of tumors are based on undifferentiated small blue round cell neoplasms, predominantly in the bones and, less frequently, in soft tissues [53].

Ewing's sarcoma has a low incidence of only 1 to 3 cases per million people per year [53]; however, the survival rate in cases of recurrence is quite low (approximately 10%). Given that the recurrence rate is approximately 30%, this disease presents an increased risk of chronic health conditions [1], [53].

Ewing's sarcoma comes from certain mutations, such as the translocation between the Ewing sarcoma RNA-binding protein 1 (EWSR1) gene on chromosome 22 and the Friend Leukemia Integration 1 (FLI1) gene on chromosome 11. The combination of these two genes leads to the expression of an abnormal protein, called Ewing's sarcoma protein, which causes defects in the differentiation process of human mesenchymal stem cells, neuroectodermal cells and Ewing's sarcoma tumor cells [54].

Despite of the aggressiveness of this cancer, the introduction of chemotherapy in the treatment has been very promising, increasing the survival rate from 10% to 75% [52]. Currently, the available treatments for this disease consist of a combination of chemotherapy, surgery, and radiotherapy [53].

2.3.4. Synovial Sarcoma

Synovial sarcoma is a rare malignant tumor which the exact cellular origin remains inadequately defined. This sarcoma constitutes 5 to 10% of all soft tissue sarcomas and is significantly more common in teenagers and young adults. The average age for the diagnosis of this sarcoma is significantly lower than that observed in other types of soft tissue sarcomas [55].

This type of cancer is classified as a neoplasm, more specifically a malignant neoplasm of mesenchymal origin, which shows characteristics of partial epithelial differentiation. This neoplasm has a high probability of local recurrence and metastases, with metastases occurring in around 50 % of patients [55]. The most prevalent sites of metastasis are the lungs and pleura. Currently, this sarcoma has been scientifically proven to occur in most areas of the body, including the viscera [56].

Synovial sarcoma is characterized by its high-grade aggressiveness, with survival rates at 5 years of approximately 60% and at 10 years of approximately 50% [55]. Typically, the bigger the tumor, the more challenging the prognosis tends to be. Furthermore, the presence of metastases might considerably impact the patient's overall condition [55], [56].

The main treatments for synovial sarcoma are the surgical resection, to the complete excision with negative margins [57]; radiation therapy, often used pre- or post-operatively to reduce recurrence risk [58]; chemotherapy, that is most effective in young patients with high-risk or metastatic disease [59]; and targeted therapies and immunotherapy, like Tazemetostat (EZH2 inhibitor) that is still emerging [60].

However, this type of cancer presents some clinical challenges, such as the high rate of metastization, the heterogeneous response to chemotherapy, local and late recurrence, late diagnosis and the lack of specific molecular therapies [57], [61].

2.4. Tumor microenvironment (TME) in OS

TME plays a fundamental role in the development and progression of cancer. Therefore, it is imperative to investigate the TME and its influence on tumor heterogeneity and growth. [62], [63]. Given that OS develops in bone tissue, its ECM is made up of a highly mineralized composition.

The modulation of the ECM and its stiffness are important factors in OS that significantly affect how the tumor grows, spreads, and responds to treatment. Acquiring a more profound comprehension of these factors is essential for optimizing therapeutic strategies and advancing patient outcomes in the management of OS.

The TME is made up of a wide variety of cells and blood vessels, providing the necessary conditions for the development and spread of both primary and metastatic tumors. It includes osteoblasts, osteoclasts, osteocytes, osteogenic cells, fibroblasts, blood vessel cells, as well as various molecules and growth factors, among other components (Fig. 2) [64], [65], [66].

Cancer development is largely influenced by two key processes: angiogenesis, which is the formation of new blood vessels from pre-existing ones, and metastasis, where the tumor spreads to distant organs through the blood or lymphatic system [67]. Patients with advanced malignant cancers have a higher chance of developing metastases of the musculoskeletal system [68]. In addition, the most frequent site of metastases is the lungs as the surviving tumor cells in the bloodstream accumulate in the alveolar capillaries. This accumulation occurs due to the larger diameter of the cells, thus leading to the formation of a secondary tumors [46].

OS cells produce factors that favor osteoclastic activity, such as Receptor Activator of Nuclear Factor- κ B Ligand (RANKL), thereby promoting the formation of osteoclasts. This intensification of bone resorption promotes the release of molecules that were previously retained in the bone matrix, such as insulin-like growth factor type 1 (IGF-1) and Transforming growth factor-beta (TGF- β), stimulating the proliferation of tumor cells [69]. OS cells produce parathyroid hormone-related protein (PTHrP), which enhances the expression of RANKL and promotes the secretion of pro-tumor growth factors by osteoblasts, including Interleukin-6 (IL-6) and Vascular Endothelial Growth Factor (VEGF). (VEGF) [46].

In addition to tumor cells and resident bone tissue cells, the TME in this type of cancer includes several other types of cells that are important for tumor progression, such as vascular cells (endothelial and perivascular), stromal cells (mesenchymal stromal cells - MSCs and fibroblasts) and immune system cells (macrophages and lymphocytes). These cell types help in the remodeling of the ECM, the formation of new blood vessels as well as cell migration [70].

On the other hand, accelerated and uncontrolled cell growth causes oxygen depletion, leading to the creation of a hypoxic microenvironment. This leads to the activation of the Hypoxia Induced Factor (HIF), which stimulates the expression of genes involved in the glycolytic pathway. Thus, OS cells need to adapt their metabolism, switching from oxidative phosphorylation to glycolysis, leading to the accumulation of lactate and acidification of the TME [71], [72]. In addition, HIF also plays a crucial role in promoting neovascularization, which consists of the formation of new blood vessels, thereby enlarging the tumor [73].

As tumors expand, they experience mechanical stress that intensifies as a consequence of increased cellular density and the concomitant stiffening of the ECM. This mechanical stress is a pivotal factor influencing both tumor proliferation and the intricate processes underlying metastasis. Therefore, it is essential to incorporate this variable in the design and refinement of cancer models, as it significantly impacts our understanding of tumor behavior and malignancy progression [67].

As the tumor mass enlarges, it induces deformation and perturbation of the adjacent healthy tissue, thereby generating resistance to further tumor expansion. This dynamic interplay subjects the tumor to mechanical stress, arising from elevated cellular density and the increased stiffness of the ECM [74]. The tissue around the tumor exerts compressive mechanical resistance on the tumor, while at the periphery, the tension is tensile. This stress induced in the tumor is stored within it as strain energy, and as a result, tumors exhibit greater stiffness than the surrounding healthy tissue [67]. OS generally has a stroma that is more rigid and dense than healthy bone tissue. This increased stiffness creates obstacles for immune cells, making it harder for them to penetrate the ECM and reach the tumor effectively [75].

The most abundant component in the TME is the tumor-associated macrophage, which can derive either from macrophages present in bone tissue or from monocytes recruited by tumor cells [76]. These macrophages can be polarized into two distinct phenotypes: M1, which secretes pro-inflammatory signals and promotes the activation of immune cells, or M2, which secretes anti-inflammatory signals and results in a reduction of immune cell proliferation. In aggressive and metastatic tumors, there is typically a higher presence of M2 tumor-associated macrophages, which is largely influenced by the tumor cells themselves [76].

Cytotoxic lymphocytes, such as T cells, are also present in the TME and can recognize foreign antigens. However, this ability is often compromised by the

immunosuppressive tumor microenvironment and reduced expression of antigens by tumor cells [77].

Given the information presented, a comprehensive study of the TME in OS is crucial for deepening our understanding of the disease and for the formulation of novel therapeutic strategies. By assimilating insights derived from TME research into clinical practice, there exists significant potential to enhance the efficacy of treatments and to strategically address the fundamental mechanisms driving disease progression, thereby ultimately improving patient prognoses.

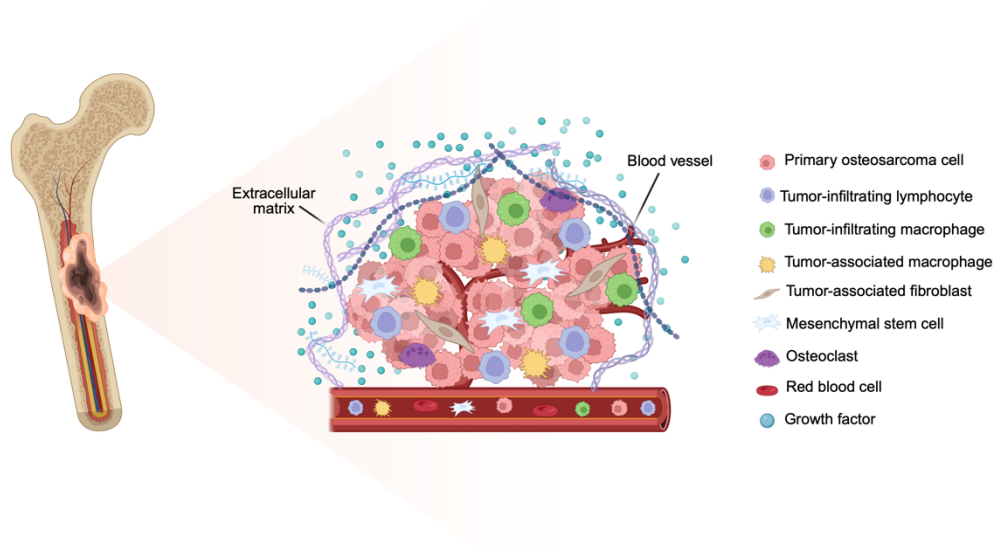


Fig. 3 - TME in OS. Created with BioRender.com

2.5. Current Approaches in OS models: Transition from 2D to 3D

The establishment of preclinical models for cancer is critically important to understand tumorigenesis and tumor progression as well as for discovering and testing new drugs and drug dosages. These models are particularly important in the context of OS, a type of cancer characterized by a high mortality rate and significant resistance to chemotherapy, highlighting the need for new drug and therapy approaches [78].

The methodologies employed to investigate cancer models can be categorized into two types: two-dimensional (2D) monolayer cultures and three-dimensional (3D) cancer

models. In the past, tumor studies primarily employed 2D culture techniques, which involved growing cancer cells on flat surfaces, under controlled conditions. While this method provided some insights, it holds limited ability to accurately model the complex interactions and spatial arrangements that occur in the TME. However, this type of cell culture model also has advantages over 3D models, such as lower costs, greater reproducibility and easier interpretation of results [79].

3D culture techniques have emerged as a more effective approach, as they allow researchers to observe and study tumors in a setting that more closely resembles their natural environment, enabling a better understanding of tumor behavior and interactions [79]. Additionally, 3D cell culture reduces the need for animal experimentation, as the use of living organisms becomes unnecessary when such a precise simulation of an organism is available [80].

However, like 2D models, 3D models also have some disadvantages, one of the main challenges being the technical complexity and time-consuming nature of establishing and maintaining 3D cell culture. These models often require specialized equipment, materials and expertise, which can limit their accessibility and scalability in routine laboratory settings [81].

In terms of cell morphology, the 2D cell culture has cells growing in monolayers, while in the 3D cell culture cells grow in multilayers allowing their shape and polarity to be more similar to *in vivo* conditions [7]. In terms of cell proliferation and differentiation, 2D cultures exhibit faster proliferation rates compared to *in vivo* conditions; however, they demonstrate limited differentiation. In contrast, 3D models allow for a proliferation rate that is better aligned with *in vivo* conditions and promote more appropriate cell differentiation [7].

In addition, 2D models lack sufficient cell-cell interactions and face greater challenges in forming cell junctions. In contrast, 3D models facilitate strong cell-cell interactions through the exchange of ions, small molecules, and electrical signals [8].

In terms of resistance to anti-cancer drugs, 2D models exhibit weak resistance as there is no barrier to drug penetration. In contrast, 3D models show higher resistance as the formation of the ECM limits drug diffusion. Furthermore, 2D models tend to be more sensitive to anti-cancer drugs, leading to a quicker induction of apoptosis in the cells. [82].

When it comes to the heterogeneity of tumor cells, 2D models only show variability if the cells are grown in co-culture. While 3D models display a high degree of heterogeneity, with the same type of cells frequently found at different stages of the cell cycle [8].

Gene expression profiles in two-dimensional (2D) models exhibit substantial discrepancies compared to those measured *in vivo*. In contrast, three-dimensional (3D) models more faithfully mimic the *in vivo* environment [70]. Finally, in terms of nutrient, growth factor, and oxygen gradients, 2D models provide a uniform environment for all cells, whereas 3D models expose cells to varying gradients. For instance, within a spheroid, cells located at the center experience less exposure to nutrients and oxygen compared to those on the periphery [83].

2.5.1. 3D Models

Several recent advancements in the development of three-dimensional (3D) models are based on tissue engineering principles, which involve the strategic combination of cells and biomaterial scaffolds to create tissue-like constructs. These constructs aim to facilitate the regeneration of tissues or organs whose functionality has been compromised due to injury or disease [84].

There are several types of 3D models mostly used in cancer research, such as spheroids, organ-on-a-chip, hydrogel-based scaffolds, organoids, and 3D bioprinting (Fig. 3) [85].

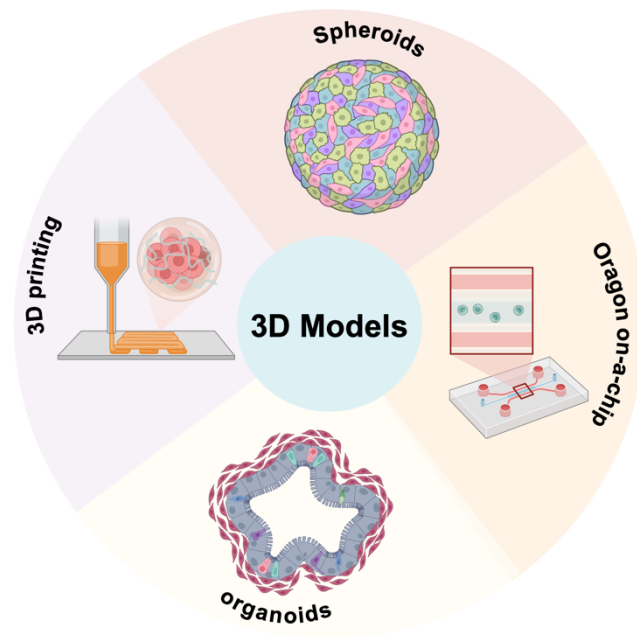


Fig. 4 - Schematic representative of different types of 3D models applied in cancer research. Created with BioRender

2.5.1.1. 3D Printing

3D bioprinting is a biomedical technology for creating complex, customizable structures and tissues by depositing layers of biomaterials. In the context of tumor tissue models, this technique offers a promising platform for the manufacture of biomimetic tumor models, which have the ability to accurately reproduce the architecture, cellular heterogeneity and microenvironment of human tumors [86]. These printing models can give rise to various cell types, components of the ECM and vascular networks, leading to a more precise study by researchers of tumor development, progression and response to drugs [87]. Some recent studies have shown the possibility of using 3D bioprinting to build OS models, breast cancer microenvironments and glioblastoma scaffolds [20], [88], [89], [90], [91], [92].

The 3D bioprinting technique allows a precise, replication of the TME through the controlled deposition of biomaterials and cells in specific layers. The abundant presence of ECM components in biomaterials contributes significantly to the functional performance of cells, for instance cell interaction with these biomaterials favours

essential processes such as proliferation, differentiation, intercellular communication, ECM synthesis and the execution of specialized biological functions [9].

Moreover, 3D bioprinting has the ability to produce complex, multicellular constructs with a high degree of reproducibility. Another advantage of this technique is that the matrix composition and stiffness can be tailored depending on the type of cancer being studied, by selecting the appropriate bioink material [9]. Bioprinted cancer models can also incorporate multiple cell types, including stromal and cancer cells within 3D constructs, thereby simulating the tailored microenvironment of this highly complex disease [9].

In this technique, selecting the most suitable matrix to accurately mimic the ECM of native tissue is of paramount importance. Natural polymer-based hydrogels are considered among the most effective biomaterials for this purpose, as they effectively replicate the tissue microenvironments characteristic of musculoskeletal tissue models. Their inherent properties facilitate the recreation of biochemical and mechanical cues, fostering a more physiologically relevant environment for cell growth and tissue development [16].

3D bioprinting is presently advancing with the goal of reconstructing various tissues within the musculoskeletal system, including bones, cartilage, muscles, tendons, and ligaments [92]. This technique necessitates the integration of properties from both biological materials and synthetic biomaterials to create functional and biocompatible constructs. By combining these diverse material properties, 3D bioprinting aims to produce sophisticated tissue architectures that closely resemble the structural and functional characteristics of native musculoskeletal tissues, [31], [93], [94], [9].

For example, Negrini et al. [95], demonstrated polyurethane scaffolds constructed by fused deposition modelling, enriched with ECM derived from human Mesenchymal Stem Cells (hMSCs), reproducing a biomimetic tumor niche, leading to greater OS cell infiltration (Saos-2). Pellegrini et al. [96] created 3D collagen-based models in order to assess tumor invasiveness and drug response in OS. Furthermore, Delgrosso et al. [97] developed an OS bioprinting model for boron neutron capture therapy studies, optimizing cell density and hydrogel composition, which is an alternative to the use of animal models.

This technique offers several advantages, including the ability to effectively mimic tumor microtissues, promote personalization and modularity, and reduce reliance on

animal models [95], [96], [97]. However, it also presents certain limitations, such as technical complexity, challenges related to cellular integration, and issues concerning reproducibility and scalability [9].

2.5.1.2. Spheroids

Spheroids consist of cell aggregates with high cell-cell adhesion designed to promote 3D cell organization. These formations display multiple resemblances to tiny tissue fragments or *in vivo* tumors, encompassing volume rise kinetics, cellular heterogeneity, and diverse cellular secretions. In spheroids, the deposition of ECM and gene expression closely resemble those of *in vivo* tumors, making them an excellent model for mimicking the characteristics of tumors [98]. Structurally, they have three distinct zones: an outer layer of rapidly proliferating cells, an intermediate layer made up of quiescent cells and a central core made up of necrotic cells. The cells in the center of a spheroid don't receive the same amount of oxygen and nutrients as the peripheral cells, and end up forming a necrotic and hypoxic core [99].

Spheroids can exhibit a high degree of heterogeneity, as they can be cultivated using various types of cells in their formation [100]. These spheroids consist of cell aggregates that self-organize through cell-cell and cell- ECM interactions, forming a 3D structure. They show greater metabolic activity, cell viability, morphological stability, gene and protein expression similar to the *in vivo* environment, as well as an increased capacity for cell differentiation [91], [101].

Multicellular spheroids are 3D cell culture models widely used in oncological research, particularly in biological studies and drug screening. These 3D models have several advantages, including ease of production, high screening capacity for anti-cancer compounds, the possibility of independent studies on culture plates and wide applicability in investigating cell growth and proliferation [13], [102]. Although spheroids have several advantages, they also have limitations, such as the difficulty in controlling the spatial organization of certain cell types, whose complex architecture can compromise reproducibility and make large-scale production difficult [9].

Despite advances in the development of 3D models, one of the main limitations of this technique is the lack of functional vascularization. Vascular networks are extremely important for the supply of oxygen and nutrients and for the removal of metabolic waste, and these factors are critical for the viability and function of cells [103], [104]. Without

adequate vascularization, diffusion limits restrict tissue thickness to just a few hundred micrometers, leading to compromised cell survival and limiting integration with host tissues after transplantation [105]. In the case of spheroids, one of the disadvantages is their spherical structure favors the establishment of an oxygen gradient, with higher concentrations in the periphery and significantly reduced levels in the nucleus. This heterogeneity in oxygen diffusion often results in necrosis in the center of the spheroid, while the peripheral cells maintain an adequate exchange of nutrient and oxygen [9].

In order to facilitate the manipulation of spheroids, magnetic spheroids have been developed, formed from cells previously labelled with magnetic nanoparticles, using a scaffold-free approach. Magnetism represents a valuable tool in the interaction with biological systems, especially when associated with magnetic nanoparticles, allowing the targeting, retention and stimulation of these structures [106].

Magnetic spheroids have additional advantages, such as high complexity and cellular heterogeneity, which contributes to better mimicking the microenvironment *in vivo*. However, they still face challenges to be optimized, including size control, morphological reproducibility and the long times required for their maturation [12].

In the case of OS, cell lines such as MG-63 and Saos-2 have been cultivated in the form of spheroids in order to investigate mechanisms of chemoresistance, hypoxia and the presence of tumor stem cells [107]. Ewing's sarcoma spheroids, normally formed from the A673 line, are used to study the interaction between tumor cells and the immune system, as well as to test immune therapies [108]. In the study of chondrosarcoma, spheroids derived from the SW1353 line allow for the analysis of intrinsic resistance to conventional therapies, such as the role of ECM and hypoxic conditions in tumor progression [109]. In addition, spheroid models of multiple myeloma, which are made up of tumor cells co-cultured with bone marrow stromal cells, allow the study of cell adhesion mechanisms and drug resistance [110]. These 3D models have become powerful tools in researching new treatments and understanding musculoskeletal tumor biology.

2.5.1.3. Organoids

Organoids are complex 3D structures derived from pluripotent or tissue-resident stem (embryonic or adult) or progenitor or differentiated cells from healthy or diseased tissues, such as tumors. They are mostly cultured in natural hydrogels, such as Matrigel, which offer a conducive milieu for cellular self-organization and the

emergence of morphological and functional traits akin to those of native organs [85]. The use of organoids as 3D models allows for the precise and organized replication of various cell types present in the human body, while spheroids offer specific advantages in studies involving molecular diffusion [9].

The production of organoids is based on the 3D culture of mammalian stem cells, associated with the sequential addition of specific growth factors that guide cell differentiation and organization [111]. Due to their remarkable ability to mimic the microarchitecture and physiological functions of the organs of origin, organoids represent a promising approach for studying development, homeostasis and human pathologies [111].

In recent years, organoids have emerged as promising 3D models for the study of musculoskeletal cancer, as they allow the preservation of tumor architecture, cellular heterogeneity and interactions with the microenvironment. According to Le Tong et al. [112], patient-derived organoids were created in order to test responses to chemotherapy drugs such as DOX and cisplatin, showing a correlation with the clinical response of patients. According to Fong et al. [113], Ewing's sarcoma organoids have been used to study specific signaling pathways, such as the EWS-FLI1 fusion, and to evaluate new targeted therapies. With regards to chondrosarcomas, which are known to be highly resistant to conventional treatments, organoids derived from human tumors have been created to investigate the influence of ECM and hypoxia on tumor behavior and therapeutic efficacy [114], [115].

2.5.1.4. Organ-on-a-chip

Organ-on-a-chip is a microfabricated cell culture platform with the aim of modelling functional units of human organs in an *in vitro* environment. To develop such platforms, it is essential to understand the anatomy and physiology of the organ of interest, identifying the essential elements for its function. Based on this analysis, the key characteristics of the functional units are determined, such as the different cell types involved, the structural organization and the physicochemical and biochemical microenvironments that regulate tissue activity [111].

These devices often have independent flow microchambers, making it possible to simultaneously cultivate different cell types and precisely control the culture microenvironment. The final manufacture of the organ-on-a-chip involves the use of

advanced microfabrication techniques [111]. Once built, these platforms are used in cell and tissue cultures with continuous perfusion, ensuring a constant supply of oxygen and nutrients. In addition, they allow for the controlled and sustained release of growth factors, providing a highly biomimetic environment for physiological and pathological study [85].

Organ-on-a-chip technology consists of an innovative platform with the ability to model musculoskeletal cancer, leading to the recreation of complex tumor microenvironments in microfluidic systems that mimic human physiological characteristics, such as perfusion, biochemical gradients and mechanical forces. In relation to OS, Chi et al. [116], created a tumor bone-on-a-chip model that integrated OS cells with bone matrix and continuous flow, allowing the study of tumor invasion and the response to chemotherapy drugs such as DOX. On the other hand, Bersini et al. [117], developed a sarcoma model on a vascularized microchip in order to investigate the interaction between tumor and endothelial cells, showing the role of the endothelium in metastatic progression.

2.5.2. Techniques for fabricating spheroids

Various methodologies have been described in the literature for obtaining spheroids, including the hanging drop technique, low adhesion plates, cell cultivation on hydrogels and magnetic levitation [9], [85]. In this work, we used the technique of cultivation on low adhesion plates with agarose coating, which provides a non-adherent surface that promotes cell grouping in suspension, favoring the spontaneous formation of spheroids.

2.5.2.1. Hanging Drop

The hanging drop technique has been widely used for the formation of cell spheroids [85], [118]. This method is based on the creation of suspended drops of culture medium containing cells, which initially form cell aggregates and then organize themselves into spheroidal structures due to the absence of an adhesion surface (Fig. 5) [9], [85]. The complete formation of spheroids using this technique requires several days of cultivation, resulting in structures that mimic tumors *in vivo* [9], [119].

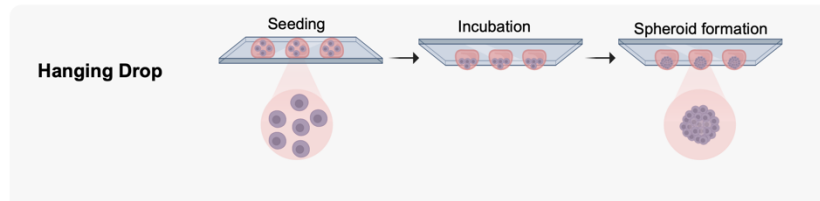


Fig. 5 - Schematic representation of the hanging drop method. Created with BioRender.com

The spheroids generated can be grown in test tubes, glass containers or multi-well plates. However, one of the limitations of this approach is related to the development and evaluation of drugs, since the volume of cell culture is reduced, making it difficult to administer therapeutic compounds accurately [119].

For example, Rimann et al. [120], used the hanging drop method to produce micromasses derived from OS cell lines (HOS, MG-63, Saos-2) and primary cells, showing that the more compact structures provide greater resistance to drugs due to lower uptake and the physical barrier associated with the microenvironment. In another study, León et al. [121], made multicellular OS spheroids using the hanging drop method and used them to test the antitumor activity of the VO-chrys complex, showing reduced viability and morphological changes in the spheroids.

2.5.2.2. Low attachment plates

The ultra-low adhesion plate technique is based on the same principle as the hanging drop technique, i.e. the formation of spheroids occurs due to the absence of an adhesive surface [9], [122]. This method uses multi-well plates coated with inert substrates that prevent cell adhesion, thus promoting cell-cell interactions and favoring the spontaneous formation of spheroids (Fig. 6) [85].

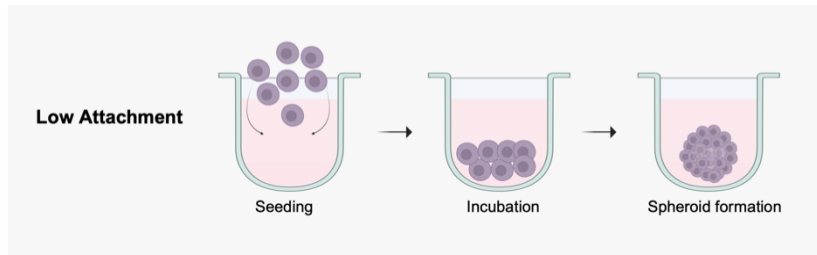


Fig. 6 - Schematic representation of the low attachment plate method. Created with BioRender

Compared to the hanging drop technique, this method has significant advantages, including the production of a greater number of spheroids, greater diversity in cell growth patterns, the formation of larger volume spheroids and the possibility of prolonged use of spheroid models [9], [122]. Furthermore, it is an approach that has been widely adopted in pharmacological evaluation trials due to its simplicity, low cost, high reproducibility and compatibility with various tumor lines [85], [123].

However, this method also presents limitations, such as the limited anchorage of spheroids within the medium, which can hinder medium exchange procedures due to the high mobility of the spheroidal structures [85], [123].

For example, Arai et al. [124], produced MG-63 OS spheroids in low attachment plates and revealed that the spheroids showed greater resistance to DOX compared to monolayer cultures, identifying the proteins associated with resistance, such as cathepsina D.

2.5.2.3. Magnetic Levitation

Magnetic levitation is an innovative approach to form spheroids, offering several advantages over conventional techniques including the high degree of control over the size and morphology of the spheroids [125], [126].

By modulating the intensity of the magnetic field, it is possible to manipulate the aggregation of magnetically labeled cells, allowing the formation of spheroids with consistent and uniform dimensions (Fig. 7). Such precision is difficult to achieve with traditional methods, in which variability in cell density and handling can result in heterogeneous spheroids [85].

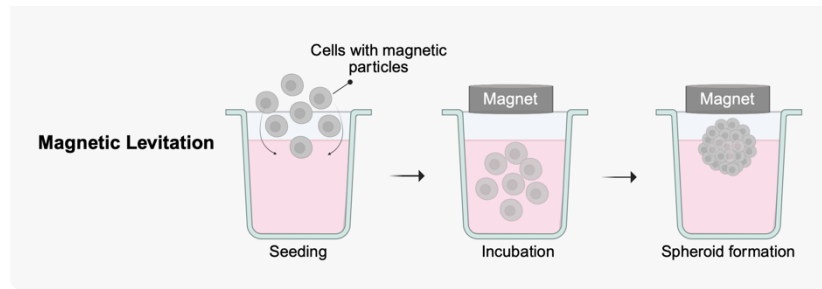


Fig. 7 - Schematic representation of the magnetic levitation method. Created with BioRender

Another relevant benefit of magnetic levitation is its ability to promote the formation of complex cellular structures that more faithfully replicate physiological conditions *in vivo*, due to the controlled cell aggregation, formation of organized structures and physiological mimicry. By remaining suspended in a liquid medium without direct contact with solid substrates, the cells are able to self-organize more effectively in 3D, thereby more accurately replicating the architecture and cellular interactions characteristics of native tissues [125], [126].

Magnetic levitation allows the controlled introduction of multiple cell populations and the creation of co-cultures, providing valuable information on the dynamics between different cell types in the tumor context [85], [126].

For example, Gaitán-Salvatella et al. [127], showed the formation of spheroids from human fetal osteoblast cells (hFOB) through the use of magnetic nanoparticles and levitation, generating stable structures with mineralized matrix deposition and expression of osteogenic markers for 14 days, demonstrating feasibility and bioactivity in bone engineering. In addition, the latest magnetic models show that spheroids promote the deposition of ECM proteins such as collagen and fibronectin, leading to a more realistic tumor microarchitecture [127], [128].

3. Materials and Methods

3.1. Cells culture

Two different types of cells were used in the present study: a human OS cell line (Saos-2) and mesenchymal stem cells (MSC).

3.1.1. Saos-2 Cell Culture

Saos-2 cells are a human OS cell line obtained from a primary OS. This cell line was purchased from Sigma-Aldrich/ Merck (ECACC) company. Initially the cells were thawed from liquid nitrogen and cultured in DMEM: Ham's F12 supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin. All cells were cultured in T-flasks, maintained under 5% CO₂ atmosphere at 37°C in the incubator.

The medium was replaced every 2 to 3 days, removing all non-adhered cells from the Tflask. Cells were trypsinized using Tryplex when they reached a confluency about 80% and transferred to a new flask, when the purpose was the growth, or to well plates, to produce the spheroids.

3.1.2. Mesenchymal stem cells (MSC) Cell Culture

The Human Mesenchymal Stem Cells used is a cell line SCC034, obtained from bone marrow. This cell line was purchased from MilliporeSigma/ Sigma-Aldrich. The cells were thawed from liquid nitrogen and cultured in alfa-MEM supplemented with 10% FBS, 1% penicillin-streptomycin. All cells were cultured in T-flasks, maintained under 5% CO₂ atmosphere at 37°C in the incubator.

The medium was replaced every 2 to 3 days, removing all non-adhered cells from the Tflask. Once the cells achieved approximately 80% confluency, the same protocol used for Saos-2 cells was followed for passaging.

3.2. Magnetic Spheroid Formation

In order to select the most adequate magnetic nanoparticles for the subsequent development of the magnetic spheroids, we tested the cell viability towards 4 different types of commercially available magnetic particles, at different concentrations: BNF_Dextran (BNF) (Micromod Partikeltechnologie GmbH), Micromer_M, COOH (Micromer) (Micromod Partikeltechnologie GmbH), Synomag_D® (Micromod Partikeltechnologie GmbH) and NanoShuttle-PL (Greiner Bio-One, n. 657841). Specifically, 15.000 Saos-2 cells were seeded into 48-well plates. The BNF, Micromer and Synomag were tested at: 10, 30, 50 e 100 μg (in total volume of 300 μL) for BNF (25 mg/mL), Micromer (50 mg/mL) and Synomag particles (20 mg/mL). For NanoShuttle, only one particle concentration was tested: 15 μg (15 μL per 150.000 cells), as recommended by the manufacturer [129]. To this, a 1:5 dilution in water was prepared for each type of particle, based on the final concentration of the stock solution and the target concentration to be tested. With these values, it was possible to obtain the values for the volume of particles (μL) to be added for each desired concentration of magnetic particles. The AlamarBlue Viability assay was performed on days 1, 7 and 14 after the particles were added to the cell monolayer, as detailed in the section 3.3.1. below.

Based on the results from these cell viability studies, we selected NanoShuttle particles for the development of the spheroids.

Spheroids were developed using the Low attachment plates method (described in the section 2.5.2.2.) with an agarose coating [130]. First, 96-well round-bottom plates were prepared by coating with agarose. A 1.5% (w/v) agarose solution was prepared in distilled water. Then, 100 μL of agarose solution was added to each well, and 70 μL was carefully removed, leaving 30 μL of agarose coating to form a rounded base in each well.

Afterwards, cells were previously seeded in 6-well plates at a cell density of 150k cells per well and incubated for 24 h. After incubation, magnetic particles were added to the cells (15 μL per well), according to the manufacturer's instructions (Figure 8). To form the spheroids, different cell numbers were tested in the agarose-coated wells: 15k, 25k, 35k and 50k of cells per well. The plates were then centrifuged at 300g for 10 minutes and incubated for 48 h (Fig. 9).

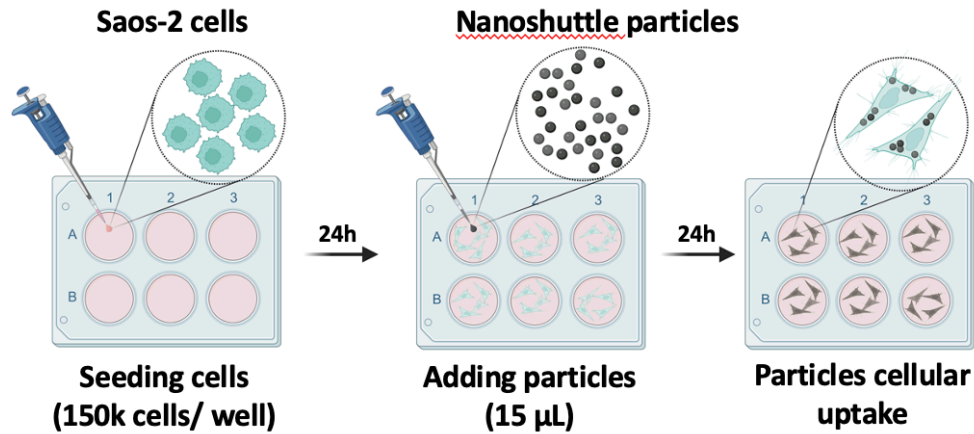


Fig. 8 - The method of incorporation of particles in cells. Created with BioRender.com.

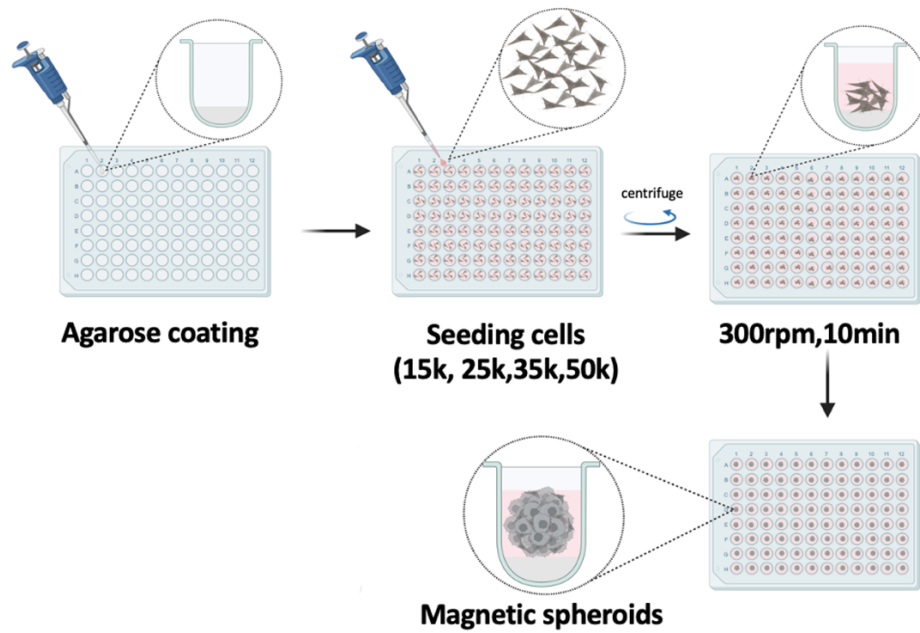


Fig. 9 - The method of formation of spheroids. Created with BioRender.com.

The produced spheroids of Saos-2 cells, but due to the poor viability and structural integrity of these spheroids it was necessary to test other possibilities.

So, we decided to combine MSC with Saos-2 to obtain mixed spheroids, in a ratio of 1:3, following a previous study by Freeman et al. [20], that showed that a 1:3 ratio between MSCs and Saos-2 cells results in spheroids with good structural integrity and

viability. For this purpose, the both type of cells were previously seeded separately in 6-well plates at a cell density of 150k cells per well. After 24 h, the magnetic particles were added to each well (15 μ L per well), according to the manufacturer's instructions. Following particle incorporation, cells were trypsinized, counted, and combined in the defined ratio 1:3 ratio. Spheroid formation was then carried out using the same protocol previously described. This approach enable improve spheroid handling for viability tests and microscopic analysis, while supporting the development of a more robust and physiologically relevant 3D tumor model.

3.3. Characterization of the Spheroids

3.3.1. Cell Metabolic activity assay

The metabolic activity of cells exposed to different types and concentrations of magnetic nanoparticles was quantitatively evaluated using two complementary viability assays: the AlamarBlue assay for monolayer cultures and the CellTiter luminescent assay for spheroids, according to the manufacturer's instructions.

AlamarBlue is a resazurin-based redox indicator that penetrates living cells and is reduced inside them by metabolic enzymes. This reduction converts the dye from its oxidized, non-fluorescent to a reduced form (fluorescent or colored), which can be measured by spectrophotometry or fluorimetry. This assay is used for cells because the dye easily penetrates monolayer cells [131]. At each pre-determined time point, the cells were washed with PBS solution before a 4 h incubation in a mixture of pre-warmed culture medium and 10% (v/v) AlamarBlue at 37 °C and 5% CO₂ atmosphere, protected from light. Then, the supernatant was transferred to a new 96-well black opaque plate and the fluorescence read at excitation and emission wavelengths of 540 and 580 nm. Three wells of each condition were analyzed, and the fluorescence readings were performed in triplicate.

The CellTiter luminescent cell viability assay was used to evaluate the metabolic activity of the spheroids. This assay quantifies intracellular ATP, an indicator of metabolically active cells, through a luminescent reaction catalyzed by luciferase. Due to its high sensitivity and efficient reagent penetration, it is particularly suitable for dense 3D cultures, such as spheroids, where dye-based assays may be less effective [132]. The spheroids were incubated in a mix of 1:1 of CellTiter reagent and culture medium

for 1h at room temperature (RT) under agitation. Following incubation, the supernatant was transferred to a new 96-well white opaque plates and the fluorescence was measured using excitation and emission of all wavelengths. Three wells of each condition were analyzed, and the luminescence readings were performed in triplicate.

3.3.2. Live Dead assay

Live/Dead assay was performed to assess cellular viability of spheroids. The spheroids were incubated for 15 min with a solution of calcein AM (2 $\mu\text{g/mL}$) and propidium iodide (3 $\mu\text{g/mL}$) in PBS at 37°C. Afterwards, spheroids were washed with PBS and visualized under a fluorescence inverted microscope (Nikon Corporation Healthcare Business Unit. *Eclipse Ci/Ni Series Upright Microscopes*. Nikon Europe B.V., 2024.) and confocal microscope (LEICA Microsystems. Inverted microscope *Leica DMI8*. Leica Microsystems). In this microscope we obtain a green (viable cells) and a red (dead cells), that provide the merge image of spheroid, and a brightfield image.

3.4. Magnetic Stimulation of the spheroids

Magnetic stimulation of spheroids was performed using a magnetic bioreactor (MICA Biosystem) aiming to promote controlled mechanical stimulation and/or cell orientation that can influence cells behavior and ECM deposition.

Firstly, the magnetic spheroids, produced as described above, were transferred to the magnetic bioreactor. It was ensured that the spheroids were aligned along the axis of the magnetic field, corresponding to the zone of maximum interaction. Following proper position, the magnetic field was applied with a displacement of 5cm and a frequency of 2 Hz for a duration of 7 days. Throughout the stimulation period, the system was maintained inside the incubator (37°C, 5% CO₂), and the spheroids were monitored for viability, morphology, and displacement behavior.

After the magnetic stimulation was completed, the spheroids were removed for analyses of viability.

3.5. Embedding Magnetic Spheroids in a 3D Matrix (Collagen Hydrogel) and Evaluating Cell Viability and proliferation

Magnetic spheroids were embedding into Matrigel hydrogel solution (Matrigel® Matrix, ref.356237). μ -Slide Angiogenesis plates were used to embed and culture the spheroids into ColMA hydrogel. For 3D spheroid embedding, a first layer of Matrigel (5 μ L) was made by putting in the incubator for 5min, performing a semi-crosslinking. Then magnetic spheroids were placed on the top of the first layer, as much as possible in the center of the well, and a second layer (5 μ L) was formed at the same conditions, during 10min. Then, culture media was added to each well and the samples were incubated for 14 days. The culture medium was replaced every 2 to 3 days. After 1d and 7d of spheroids embedding, a Live/Dead cell assay was performed for viability assessment. After spheroids embedding, cell spheroid invasion was monitored and imaged using a light microscope. Image acquisition was performed every day until the third day and then at 7, 10, and 14 d of culture. The invasion is defined as:

$$Invasion = \frac{A_t - A_0}{A_0}$$

In which A_t is the area of the spheroids at day 1, 7 and day 14, and A_0 is the area of spheroids at day 0. The brightfield images will be employed to measure the spheroid area at different time points through the ImageJ software.

3.5.1. Immunofluorescence of Ki-67

Furthermore, the aggressiveness and progression of the OS spheroids was evaluated by utilizing Ki-67 immunofluorescent staining. The spheroids were washed three times with PBS, before and after fixation with 10% (v/v) neutral buffered formalin overnight and kept in PBS at 4°C until usage. Subsequently, the spheroids were incubated with a 0.2 % Triton-X100 in PBS solution and incubated for 10 minutes at RT. After spheroids were washed three times with PBS and blocking step was performed used 2% BSA in PBS solution, during 2h at RT. Then, spheroids were washed with PBS and incubated overnight at 4°C with the primary antibody (Rabbit anti-human Ki-67, 1:1000) diluted in 0.1% BSA in PBS solution, followed by 1h incubation at room temperature with the secondary antibody (donkey anti-rabbit Alexa Fluor 488, 1:200).

The immunostained samples were then analyzed by fluorescence inverted microscope and confocal microscope.

Ki-67 is a well-known marker for cellular proliferation, allowing to identify and quantify the proportion of dividing cells within the spheroids. By assessing the expression levels of Ki-67, we can gain insights into the growth dynamics and aggressive characteristics of the cancer cells. This information is crucial for understanding how magnetic fields may influence the behavior of these OS spheroids, potentially affecting their invasiveness and overall aggressiveness in a tumor microenvironment.

3.6. Study of Drug Penetration within Magnetic Spheroids

The diffusivity of DOX was assessed in two different conditions: in magnetic spheroids subjected to magnetic stimulation, and in magnetic spheroids maintained without magnetic stimulation. By comparing drug diffusion rates between these conditions, the study aimed to evaluate how the surrounding matrix influences the distribution and therapeutic efficacy of DOX OS cells. This analysis contributes to a better understanding the role of both the ECM and magnetic field exposure in modulating drug delivery and potential treatment outcomes.

DOX was dissolved in the culture medium and added to the wells containing the spheroids. DOX penetration into the spheroids was evaluated over time by fluorescent microscope and confocal, Furthermore, the cytotoxicity of DOX was assessed by testing various drug concentrations through viability assays, including live/dead staining and the AlamarBlue assay, following the protocol described above.

3.7. Statistical Analysis of Data

Data is presented as the mean $\pm$ standard deviation (SD) values. All experiments were run in technical triplicates (N=3), unless stated otherwise, each consisting of technical replicates. Statistical analysis was performed using t-student tests, in GraphPad Prism 10 software (GraphPad software Inc., La Jolla, USA). Data was statistically significant when p-values less than 0.05% were obtained (95% confidence level intervals, *p<0.05).

4. Results and Discussion

4.1. Selection of the most adequate magnetic particles - Saos-2 cells viability towards different concentrations

In pursuit of developing 3D magnetic spheroids, the initial step involved combining magnetic particles with Saos-2 cells. To do this, the first step was to select the most adequate magnetic particles to be used. Initially, the feasibility of integrating four distinct magnetic particles including BNF, Micromer, Synomag and NanoShuttle was assessed at different concentrations (10, 30, 50 and 100 μg). The Alamar Blue assay was performed to confirm the non-cytotoxic effect magnetic nanoparticles on days 1, 7 and 14.

The results demonstrated that cells maintained high viability across all concentrations of Micromer and Synomag particles over a two-week culture period (Fig. 10. B, C). For BNF particles, high viability was observed at concentrations of 10, 30, and 100 μg ; however, a decrease in viability was noted at the 30 μg concentration over 14 days (Fig. 10A). In the case of Nanoshuttle particles, cell viability remained elevated (approximately 130%) during 7 days, followed by a slight decrease (Fig. 10D). This reduction may be attributed to increase cell proliferation, which likely resulted in limited available surface area for cell adherence in the well plate [133].

In the case of BNF particles, Patil et al. [134], reported that *in vitro* and *in vivo* studies using dextran-coated iron oxide nanoparticles demonstrated sustained high cell viability at concentrations up to approximately 1 mg/mL, with significant cytotoxic effects observed only at higher concentrations. These findings are in agreement with our results, which showed that the concentrations tested did not affect cell viability over the 14-day period. On the other hand, according to the graph in figure 10B and C, the Micromer and Synomag particles there were no significant changes in viability. Novoselova et al. [135], used the CellTiter Blue assay on HeLa cells exposed to various concentrations of Synomag (Fig. 10A). Their results indicated no significant decrease in cell viability, even at the highest concentrations tested after 24 h of exposure, as indicated in the viability graph.

While all particle types demonstrated the ability to support cell viability, NanoShuttle particles showed most consistent and robust performance over time. Based on this,

NanoShuttle was selected for subsequent studies as the most promising option due its sustained support of cell viability.

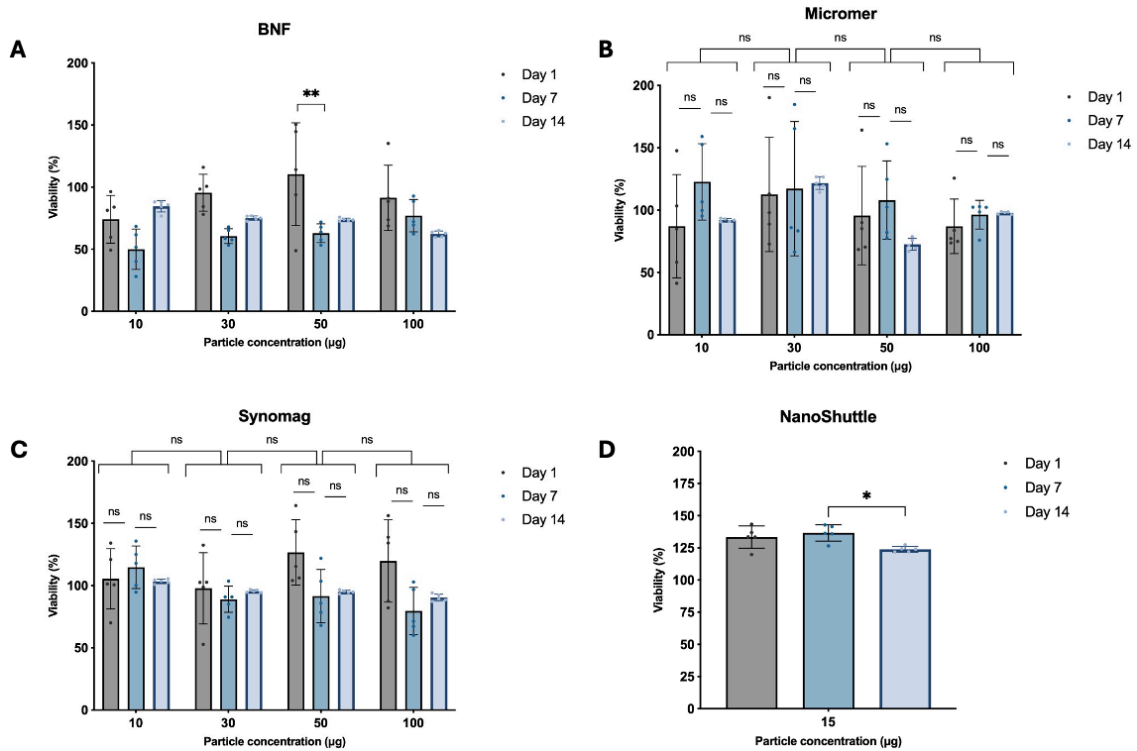


Fig. 10 - Cell viability of Saos-2 cells treated with different types of particles at different concentrations, measured by the AlamarBlue assay. Panels show viability for cells after incorporation with (A) BNF particles; (B) Micromer particles; (C) Synomag particles; and (D) NanoShuttle particles. (N=6). Symbols denote statistical differences, * for $p<0.05$, and ** for $p<0.01$.

4.2. Spheroid formation

4.2.1. Incorporation of Nanoshuttle particles in cells

To confirm the incorporation of Nanoshuttle particles into the cells, Saos-2 cells were observed 24 h after particle incubation using a ZOE microscope at 4x magnification. The presence of magnetic particles inside the cells was confirmed, as indicated by the yellow arrows in figure 11.

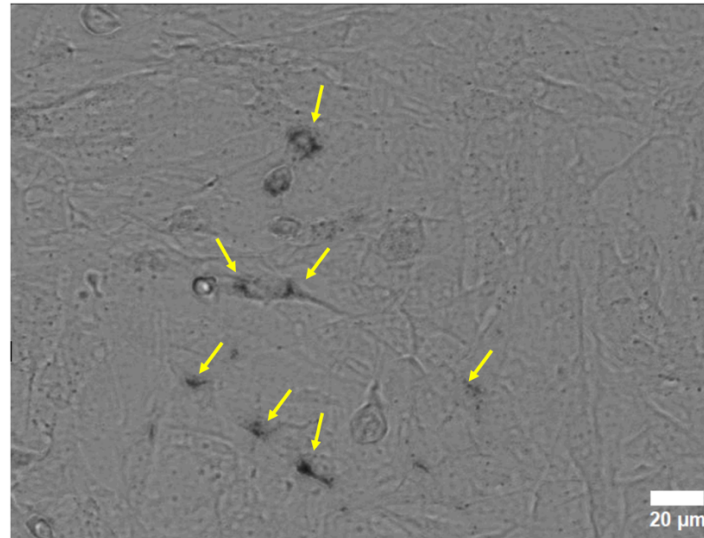


Fig. 11 - Cellular uptake of Nanoshuttle particles in Saos-2 cells. Yellow arrows indicate the presence of Nanoshuttle particles inside the Saos-2 cells after 24h of incubation.

4.2.2. Development of the magnetic OS spheroids

4.2.2.1. Spheroid formation of Saos-2 cells with NanoShuttle

To produce magnetic OS spheroids, Saos-2 cells were first incubated with Nanoshuttle particles for 24 h. After particle incorporation, the cells were trypsinized and seeded in Low-attachment plates, coated with agarose, at four different cell densities: 15.000, 25.000, 35.000 and 50.000 cells/well. Control spheroids were generated without magnetic nanoparticles. Spheroids morphology was observed under a microscope using brightfield, as shown in figure 12.

As previously described, Saos-2 cells are capable of self-aggregating within 24 h of seeding [136]. However, in this study, spheroids were allowed to form for 48 h to achieve a more homogeneous, rounder structure with improved structural integrity, except for the initial trial, where spheroids were formed over 24 h. Once formed, these spheroids had a circular shape with reasonably well-defined boundaries. Their size was directly related to the cell density used and the duration of the culture.

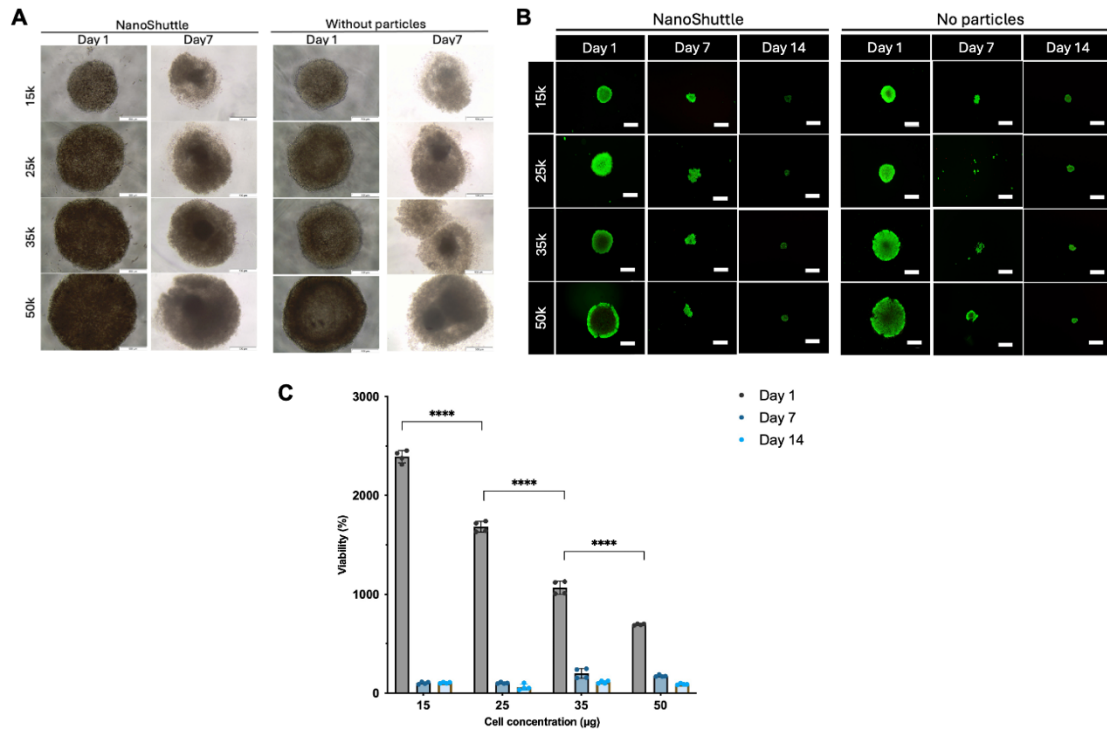


Fig. 12 - Production of Saos-2 spheroids with NanoShuttle at different cell densities: 15.000, 25.000, 35.000, and 50.000 cells/well. (A) Brightfield microscopy images, at days 1 and 7 (Scale bars 500 µm). (B) Live/Dead staining (green: live cells, red: dead cells) at days 1, 7 and 14 (Scale bars 100 µm). (C) Viability of spheroids using the CellTiter assay. (N=6). Symbols denote statistical differences, ****p<0.0001. There are significant differences between the days for each cell concentration condition. (not represented in the image for simplification).

At all four cell densities, well-defined and compact spheroids were observed 24 h post-seeding. As shown in figure 12A, on day 1, the spheroids exhibited a circular morphology with reasonably boundaries and the size was directly related to the cell density of spheroids. However, on day 7, structural disintegration of the spheroids was evident, characterized by cellular growth and invasion of the cells around the spheroid. Additionally, at day 7 a necrotic core was observed in the spheroids at all cell densities (Fig. 12A). This cellular behavior aligns with the rapidly proliferating and invasive behavior of cancer cells. These findings are consistent with the study by Sagheb et al. [137], who demonstrated necrosis within the core of OS spheroids through the Live/Dead assays, indicating a loss of structural integrity after several days in culture.

To assess the cell viability within the spheroids, both the Live/Dead cell assay and the CellTiter assay were performed over a 14-day period.

In the context of the Live/Dead assay, despite the predominance of viable cells observed in figure 12B, a significant degree of disintegration and loss of structural integrity was evident. This degradation was particularly pronounced on day 7 and became increasingly conspicuous by day 14.

Although there are still no studies describing the formation of Saos-2 cell spheroids in the presence of NanoShuttle, Chaweewannakorn et al. [138] showed that pre-incubation with NanoShuttle enhances the robustness of muscle cells (C2C12) spheroids, allowing them to be manipulated without comprising their integrity. Additionally, Hou et al. [139] reported that NanoShuttle facilitates faster spheroid formation.

Taken together, these findings suggest that NanoShuttle incorporation may improve spheroid formation dynamics and mechanical stability, highlighting its potential for applications requiring durable and manipulated 3D cell cultures.

Regarding the CellTiter assay, a marked reduction in spheroid cell viability was observed from day 1 to days 7 and 14, which corresponded with a progressive decrease in spheroid size (Fig.12C). Notably, when comparing spheroids formed with and without NanoShuttle, a significantly increase of viability was observed on day 1 in cells with NanoShuttle at all cell densities. This can be clearly seen in figure12C, where the data were normalized to the viability of spheroids formed without NanoShuttle, allowing direct comparison.

The main objective of this experiment was to determine the optimal cell density for the formation of viable spheroids. Results indicate that lower initial cell densities yielded higher viability, particularly on day 1, with a linear inverse correlation between cell number and viability. These findings suggest that lower cell densities are preferable due to their capacity for generating viable spheroids. Despite these results, it was decided to continue testing the four concentrations in order to have more robust data for selecting the optimal density.

4.2.2.2. Spheroid formation of Saos-2 cells with Micromer particles

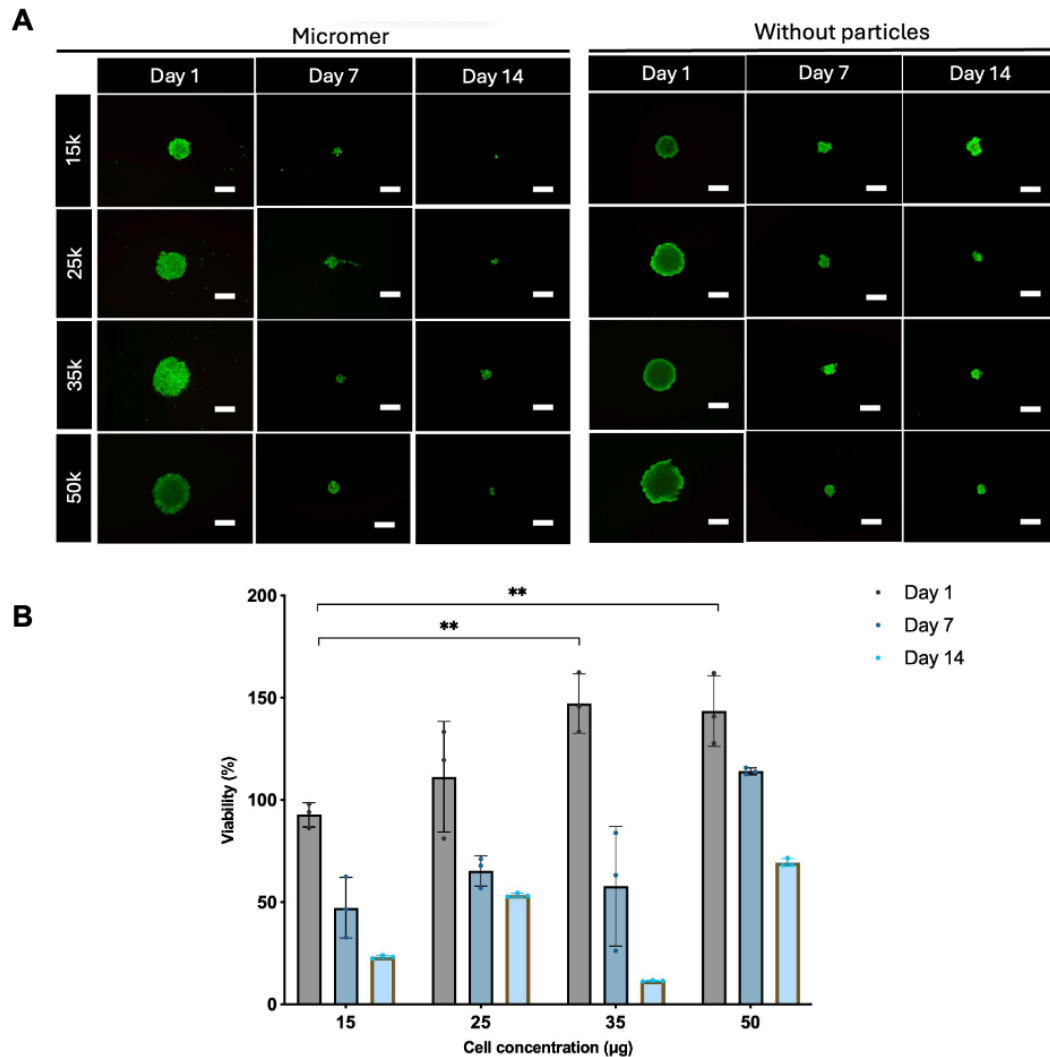


Fig. 13 - Production of Saos-2 cells spheroids with Micromer at different cell densities: 15.000, 25.000, 35.000, and 50.000 cells/well. (A) Live/Dead staining (green: live cells, red: dead cells) at days 1, 7 and 14 (Scale bars 100 µm). (B) Viability of spheroids using the CellTiter assay. (N=6). Symbols denote statistical differences, ****p<0.0001. There are significant differences between the days for each cell concentration condition. (not represented in the image for simplification).

In order to exclude the possibility that the low cell integrity and viability was due to the NanoShuttle, spheroids were produced with alternative types of magnetic particles, namely MNP from Micromer. These particles were chosen considering the cell viability tests carried out previously (Fig. 10) on various types of magnetic particles, where the Micromer particles proved to be the most viable after the NanoShuttle particles. In this assay, control spheroids without magnetic particles were also produced for comparison.

As before, cell viability was assessed using Live/Dead staining and the CellTiter assay. Similar to the results observed with NanoShuttle particles, a high number of live cells were observed, however, spheroids exhibited poor structural integrity (Fig. 13A) and reduced overall cell viability (Fig. 13B).

As observed in the previous experiment, the spheroids disintegrated and their size decreased considerably, as shown in figure 13B. Cell viability significant decrease between day 1 and the later times points (days 7 and 14). Although this decrease is not as marked because the Micromer particle does not have as high a viability value as the NanoShuttle on the first day, which is around 2000% for the NanoShuttle and around 100% for the Micromer, at a concentration of 15 μ g.

These results suggest that the observed loss of spheroid integrity is not related to NanoShuttle particles and may instead be related to the inherent proliferative behavior of the Saos-2 cells or limitations associated with spheroid size and density under the tested conditions.

In contrast to the previous experiment, cell viability in this assay increased with increasing cell concentrations. However, when comparing the cell concentration of 35.000 with that of 50.000 cells per well, a greater viability was seen at the 35.000 cells per well concentration, making it a good target of choice. At this stage, it was also decided to continue with the four different concentrations.

4.2.2.3. Spheroid formation of Saos-2 and MSC cells

In an effort to improve the structural integrity and viability of Saos-2 cells spheroids, it was decided to produce spheroids by combining MSCs with Saos-2 cells. To determine the optimal cell ratio for spheroid production, two ratios were tested, 1:3 and 1:5 of MSCs and Saos-2 cells, respectively. These ratios were evaluated across 4 different cell densities: 15.000, 25.000, 35.000, and 50.000 cells/well. According to Freeman et al. [20], integrated spheroids can be successfully formed using an MSCs:Saos-2 ratio of 1:3. Based on this, the previously reported ratio was tested with a lower number of MSCs cells, in order to maximize the number of cancer cells within the spheroid.

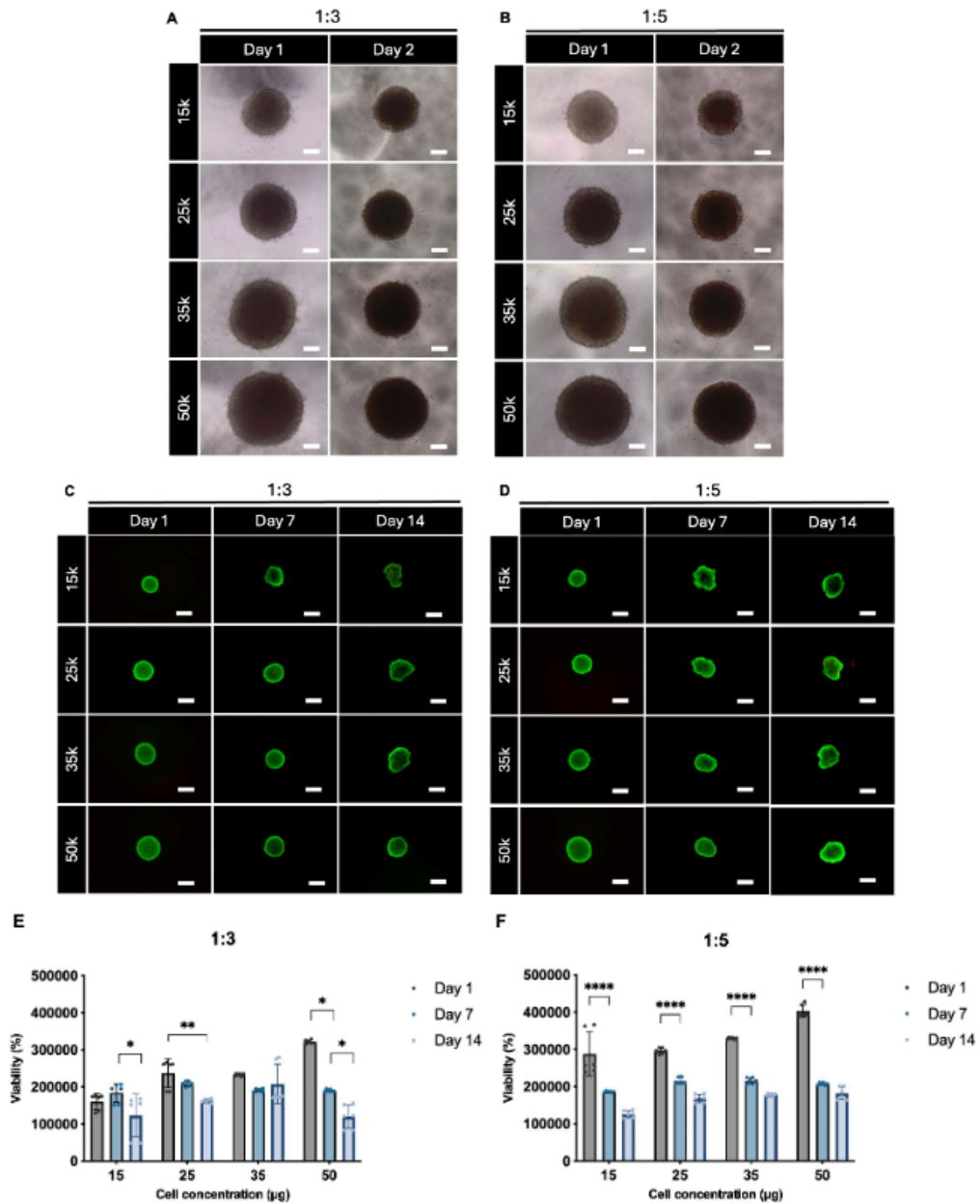


Fig. 14 - Spheroid formation using MSCs and Saos-2 cells in low attachment plates coated with agarose, seeded at different cell densities: 15.000, 25.000, 35.000, and 50.000 cells/well. Brightfield images of spheroids formed with MSCs: Saos-2 cells (A) ratio 1:3, and (B) ratio 1:5, at days 1 and 2 of formation (Scale bars 100 μ m). Live/Dead staining (green: live cells, red: dead cells) of spheroids formed with MSCs: Saos-2 cells (C) ratio 1:3, and (D) ratio 1:5, at days 1, 7 and 14 (Scale bars 100 μ m). There are significant differences between the cell concentrations. Viability of spheroids to the (E)ratio 1:3 and (F) ratio 1:5 MSCs and Saos-2 cells. (N=6). Symbols denote statistical differences, * p <0.05., ** p <0.01, and **** p <0.0001. There are significant differences between the cell concentrations conditions. (not represented in the image for simplification).

Thus, the spheroids were produced using the previously described method, and monitored during 48 h of spheroid formation using a microscope. As shown in figure 14A

e 14B, both MSC:Saos-2 cell ratios tested were able to produce spheroids with good apparent integrity and a rounded shape, as desired. As expected, spheroid size increased with increasing cell density. In addition, the presence of a necrotic core in the spheroids was also noted. Freeman et al. [20], showed in their study the successful formation of spheroids using a 1:3 ratio MSCs:Saos-2 cells ratio, with good structural integrity and viability over the several days. Similarly, our data confirm the presence of a necrotic core, which became more prominent at higher cell densities.

Following this, spheroids were evaluated for integrity and cell viability tests at 24h, 7 days and 14 days of culture using Live/Dead assay. As shown in figures 14C e 14D, for both ratios, most cells remained viable over time, with a minimal number of dead cells detected. The spheroids also maintained their structural integrity and the size over time.

Regarding the CellTiter assay, cell viability remained stable over the days for both ratios, with only a little decrease over time, as shown in figure 14E, for the 1:3 ratio and 14F, for the 1:5 ratio. Notably, a modest increase in viability was observed at certain points for the 15k and 35k cell densities within the 1:3 ratio condition.

Based on these results, the 1:3 MSC: Saos-2 ratio was selected for subsequent experiments. This decision was supported by its more stable viability profile over time and better integrity observed on day 7 when compared to the 1:5 ratio. Additionally, the 50k cell density from further analysis, as these spheroids developed a large necrotic core, leading to insufficient nutrient and oxygen diffusion and a more pronounced decline in viability over time.

4.2.2.4. Spheroid formation of Saos-2 and MSC cells with NanoShuttle

Given the successful formation of spheroids using a 1:3 ratio of MSC and Saos-2, we tested the formation of spheroids under the same mixture and ratio in the presence of NanoShuttle. Two experimental conditions were tested: (1) NanoShuttle particles added only in MSC cells, and (2) NanoShuttle particles added only in Saos-2 cells. Control spheroids were also produced, without magnetic particles.

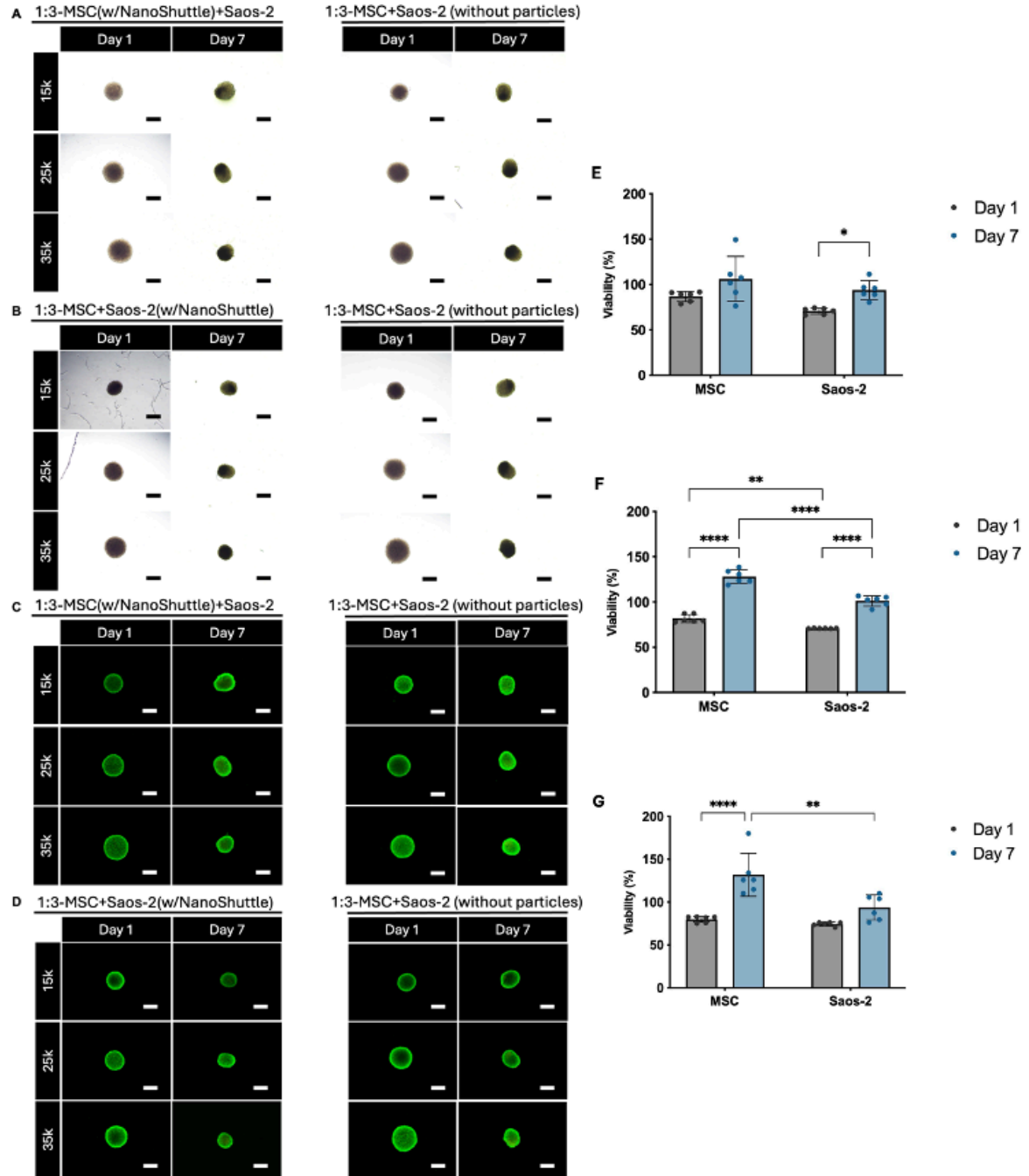


Fig. 15 - Spheroid formation using MSCs and Saos-2 cells with NanoShuttle, in low attachment plates coated with agarose, seeded at different cell densities: 15.000, 25.000 and 35.000 cells/well. Brightfield images of spheroids formed with MSCs: Saos-2 cells in a ratio of 1:3, (A) with the presence of Nanoshuttle only in MSCs, (B) with the presence of NanoShuttle only in Saos-2, at days 1 and 2 of formation. Both conditions have a control group without the presence of NanoShuttle (Scale bars 100 μ m). Live/Dead staining (green: live cells, red: dead cells) of spheroids formed with MSCs: Saos-2 cells, in a ratio of 1:3, (A) with the presence of Nanoshuttle only in MSCs, (B) with the presence of NanoShuttle only in Saos-2, at days 1 and 2 of formation. Both conditions have a control group without the presence of NanoShuttle (Scale bars 100 μ m). Viability of spheroids to the (E) concentration of 15.000 cells per well, (F) concentration of 25.000 cells per well and (G) concentration of 35.000 cells per well. Values normalized by the control group values. (N=6). Symbols denote statistical differences, * p <0.05., ** p <0.01, and **** p <0.0001. There are significant differences between the cell concentrations conditions. (not represented in the image for simplification).

Spheroids from both conditions were observed using microscopy (Fig. 15A and 15B) on days 1 and 7 after formation. All conditions and cell densities showed good structural integrity, despite the slight decrease in spheroid size from day 1 to day 7.

To compare the cellular integrity of the spheroids between conditions for all the cell densities, the Live/Dead cell assay was performed. As shown in figures 15C and 15D, despite a slight decrease in the size of the spheroids observed in both conditions, improved integrity was noted compared to the previous experiment.

To assess spheroid cell viability, the CellTiter assay was performed. Figures 15E, 15F and 15G show the viability results of conditions where NanoShuttle was added to MSCs cells, to Saos-2 cells, as well as for the control group without magnetic particles, across three seeding densities (15.000, 25.000, and 35.000 cell/well). In all, an increase in viability from day 1 to day 7 was observed. This increase was more pronounced when NanoShuttle was added to MSCs cells but was also notable in Saos-2 cells.

Patents such as US2020/0283733A1 and WO2017/207737A1 report that densities of between 5000-15.000 cells per well (96 well), ideally 10.000 cells, optimize the formation of spheroids in various tumor models, offering a balance between uniform size and cell viability. However, in this study, it was decided to proceed with a cell density of 35.000 cells/well. Despite the lack of scientific studies for these types of cells, the well-established values via cell density permutation confirm that this amount falls into the effective range for forming compact spheroid's structure.

4.3. Magnetic stimulation of the spheroids

The magnetic bioreactor was used for magnetically stimulating the spheroids obtained under the two conditions previously tested using a 1:3 ratio of MSC:Saos-2 cells, in two different conditions: 1) NanoShuttle particles added only in MSC cells, and (2) NanoShuttle added only in Saos-2 cells. These conditions were selected for further testing as no major differences in performance were observed between them. Spheroids from both groups were subjected to magnetic stimulation for 7 days. Control spheroids were also prepared and placed in the incubator without magnetic stimulation. The bioreactor applied a magnetic field to the samples at a frequency of 2 Hz and a

displacement of 5 cm. After 3 and 7 days of stimulation, cell viability assays and Phalloidin staining were performed to evaluate cellular organization and viability.

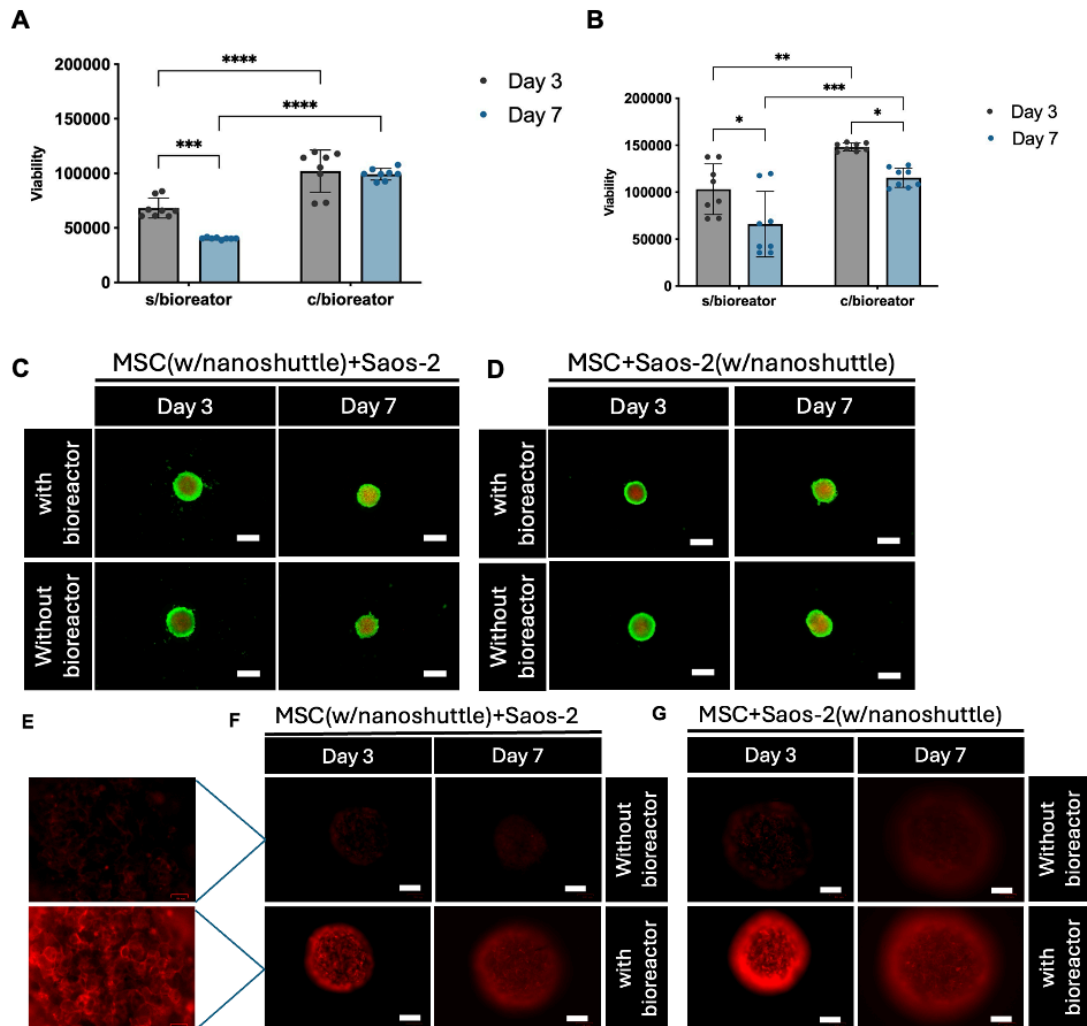


Fig. 16 - Spheroid formation using MSCs and Saos-2 cells with NanoShuttle, in a 1:3 ratio in low attachment plates coated with agarose, seeded at a cell density of 35.000 cells/well. Two conditions were tested: the presence of magnetic stimulation by a bioreactor and the absence of stimulation. Viability of spheroids (A) with the presence of Nanoshuttle only in MSCs and (B) with the presence of Nanoshuttle only in Saos-2 cells. Live/Dead staining (green: live cells, red: dead cells) of spheroids formed with MSCs: Saos-2 cells, (A) with the presence of Nanoshuttle only in MSCs and (B) with the presence of NanoShuttle only in Saos-2, at days 1 and 2 of formation. (Scale bars 100 µm). Phalloidin staining of spheroids formed with MSCs: Saos-2 cells, (A) with the presence of Nanoshuttle only in MSCs and (B) with the presence of NanoShuttle only in Saos-2, at days 1 and 2 of formation. (Scale bars 100 µm). Values normalized by the control group values. (N=6). Symbols denote statistical differences, *p<0.05., **p<0.01, ***p<0.001. There are significant differences between the cell concentrations conditions. (not represented in the image for simplification).

The results obtained from the cell viability tests (CellTiter) are shown in figures 16A and 16B. The Live/Dead staining results are presented in Fig.16C and 16D and the Phalloidin staining in figures 16E, 16F and 16G.

In figure 16A and B, an increase in cell viability was observed for both conditions in spheroids under magnetic stimulation by the bioreactor. In contrast, a decrease in viability from day 3 to day 7 was noted in spheroids not exposed to magnetic stimulation. Notably, in the condition where NanoShuttle particles were incorporated only into MSCs cells and exposed to magnetic stimulation, it was observed that viability remained constant over the time.

These results suggest that the application of magnetic stimulation contributes to the maintenance and enhancement of spheroids viability. These findings are in line with the study of Lin et al. [140], who compared a static culture and dynamic culture in a bioreactor for spheroids of NK-92MI cells. Their study demonstrated increased apoptosis in the nuclei of spheroids under static conditions by day 14, while spheroids culture in the bioreactor maintained higher viability and exhibited reduced apoptosis.

Live/Dead staining revealed a predominance of viable cells throughout the spheroids, with a predominance of dead cells in the core of the spheroid, which can be explained by the lack of oxygen and nutrients, a common feature in three-dimensional tumor models.

To further investigate the ECM content, Phalloidin staining was performed. As can be seen in figure 16E, F and G, there is a large increase in the ECM in the spheroids under magnetic stimulation in both conditions. Several authors, such as Kazimierczak et al. [141], support our results, showing that the use of a magnetic field in BMSCs leads to the production of higher levels of osteopontin, which is a key component of the ECM. Tuğcu et al. [142], also showed that HepG2 spheroids formed in RCCS or CSTR systems maintain high viability (> 80% up to 10 days), with diameters of 200-300 μm and functional activity, indicating that dynamic conditions promote more robust cell-ECM interactions and enhanced structural integrity. These results show that the effect of the magnetic field generates the production of more ECM, increasing the rigidity of the spheroids.

Taken together, these results demonstrate that the application of a magnetic field significantly enhances the viability, structural integrity, and extracellular matrix

production of Saos-2/MSC spheroids, underscoring the potential of magnetic stimulation to improve tumor spheroid models for more physiologically relevant studies.

4.4. Embedding Magnetic Spheroids in a 3D Matrix (Collagen Hydrogel) – invasiveness assessment

After 7 days of exposure to magnetic stimulation, the spheroids were embedded in a 3D matrix composed of ColMA hydrogel to assess their invasiveness. In this step, spheroids not exposed to the bioreactor were also kept as a control.

According to Huang et al. [143], in breast cancer models (MDA-MB-231 co-cultures) embedded in a 3D matrix, disaggregation and invasion are most pronounced on days 4 and 7, primarily occurring at the periphery of the spheroid while the central core remains relatively stable, with invading cells migrating along the spheroid contours. These observations align with our results, which show notable invasion at days 3 and 7 (Fig. 17A and B).

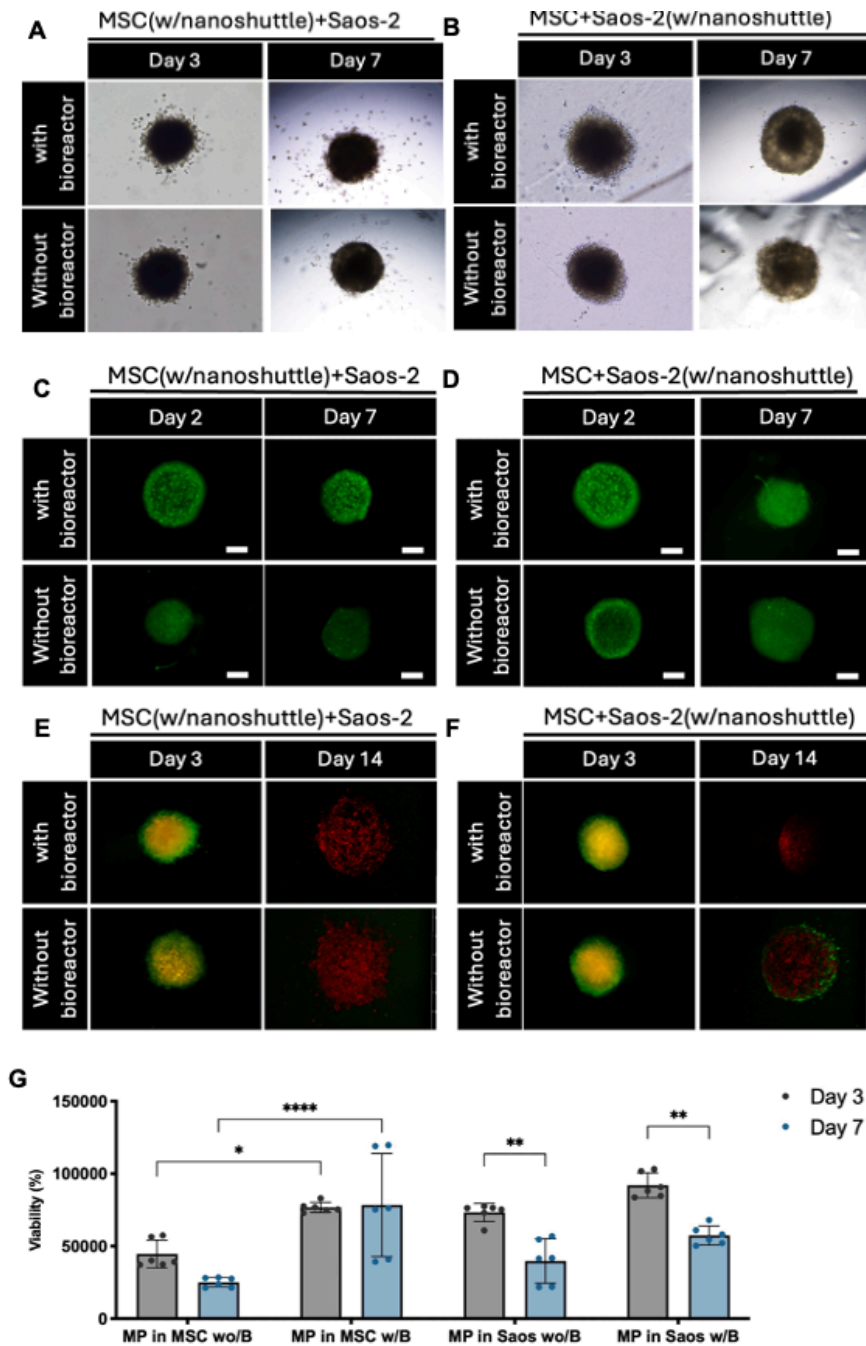


Fig. 17 - Spheroid formation using MSCs and Saos-2 cells with NanoShuttle, in a 1:3 ratio in low attachment plates coated with agarose, seeded at a cell density of 35.000 cells/well, embedding in a Matrigel. Two conditions were tested: the presence of magnetic stimulation by a bioreactor and the absence of stimulation. Brightfield images of spheroids formed with MSCs: Saos-2 cells in a ratio of 1:3, (A) with the presence of Nanoshuttle only in MSCs, (B) with the presence of NanoShuttle only in Saos-2, at days 3 and 7 of formation. (Scale bars 100 μ m). Staining with Ki-67 marker (green: cells marked with Ki-67) of spheroids formed with MSCs: Saos-2 cells, (C) with the presence of Nanoshuttle only in MSCs and (D) with the presence of NanoShuttle only in Saos-2, at days 3 and 7 of formation. (Scale bars 100 μ m). Live/Dead staining (green: live cells, red: dead cells) of spheroids formed with MSCs: Saos-2 cells, (E) with the presence of Nanoshuttle only in MSCs and (F) with the presence of NanoShuttle only in Saos-2, at days 3 and 7 of formation. The images of day 3 are from fluorescent microscope and on day 14 from confocal microscope. (Scale bars 100 μ m). (G) Viability of spheroids with the presence of Nanoshuttle only in MSCs and with the presence of Nanoshuttle only in Saos-2 cells, stimulated and non-stimulated by an extern magnetic field. Values normalized by the control group values. (N=6). Symbols denote

statistical differences, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. There are significant differences between the cell concentrations conditions. (not represented in the image for simplification). MP means magnetic particles.

Given the results shown in figures 17A and 17B, it can be concluded that magnetic stimulation using a bioreactor enhances cell proliferation and invasion in spheroids compared to those not exposed to the magnetic field. This is further supported by the increased expression of the proliferation marker Ki-67, as seen in figures 17C and 17D, which show greater labelling in the spheroids stimulated by the bioreactor. No significant changes were observed between days 3 to 7. According to Mary et al. [67], who produced spheroids of tumor cells (CT26 and glioblastoma strains) with internal magnetic particles, magnetism-induced compression resulted in an increase in proliferation, assessed by Ki 67 marking, particularly in the center of the spheroid, in addition to increased expression of MMP 9, indicative of invasive potential.

Live/Dead assays (Fig. 17E and 17F) revealed higher cell death within the spheroid cores, attributed to tumor necrosis caused by hypoxia and nutrient deprivation in the spheroid center, in day 2. On the other hand, a predominance of cell death was observed on day 14, especially in the stimulated samples, which may suggest a long-term therapeutic effect of magnetic stimulation. Peluso et al. [144] investigated the effects of magnetic stimulation on periodontal ligament stem cells, observing significant changes in cell viability after 14 days of exposure. The results indicate that magnetic stimulation can induce cellular effects that manifest after specific periods, such as day 14.

Regarding overall cell viability, figure 17G shows greater viability in the spheroids exposed to magnetic stimulation compared to the unstimulated spheroids. Although viability decreased from day 3 to day 7, in all conditions, there is greater viability in the stimulated spheroids. It should also be noted that in MSCs cells with NanoShuttle exposed to magnetic stimulation, showed stable viability from day 3 to day 7. According to Izzo et al. [145], their study showed that the presence of the magnetic field positively affected the cell viability and metabolic activity of 3D SH SY5Y cells, suggesting a biostimulatory effect.

In summary, magnetic stimulation via the bioreactor significantly enhances spheroid cell proliferation, invasion, and Ki-67 expression, while maintaining higher overall cell viability despite necrosis in the core. These findings highlight the potential of magnetic spheroids and magnetic field exposure to support the replication of proliferation

and invasiveness that characterize this tumor, thus providing better model for future cancer research and therapeutic development.

4.5. Study of drug penetration within magnetic spheroids

After 7 days of magnetic stimulation exposition, the spheroids were exposed to DOX to evaluate drug penetration into the spheroids. Two concentrations were tested: 50 nM and 100 nM. Cell viability assays and Live/Dead staining were performed on days 2 and 7 post-treatment. In the case of the Live/Dead cell assay, only the PI (red) reagent was added to the sample, as this shows the dead cells of the spheroid, while the green colour belongs to the cancerous cells of the spheroid.

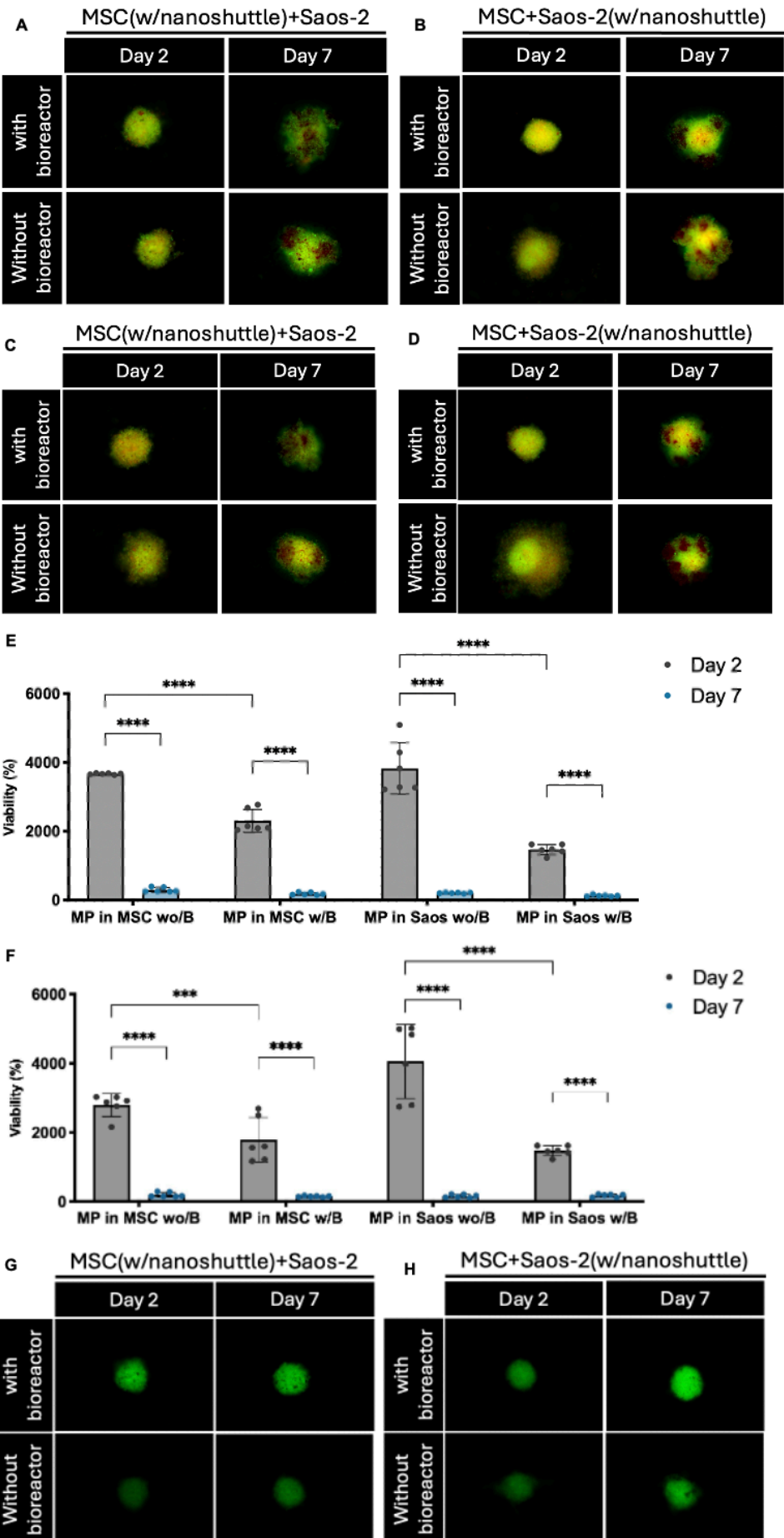


Fig. 18 - Spheroid formation using MSCs and Saos-2 cells with NanoShuttle, in a 1:3 ratio in low attachment plates coated with agarose, seeded at a cell density of 35.000 cells/well, treated with DOX. Two conditions were tested: the presence of magnetic stimulation by a bioreactor and the absence of stimulation. Live/Dead staining (green: live cells, red: dead cells) of spheroids formed with MSCs: Saos-2 cells, treated with a drug concentration of 50 nM, (A) with the presence of Nanoshuttle only in MSCs and (B) with the presence of NanoShuttle only in Saos-2, at days 2 and 7 of formation. (Scale bars 100 μ m). Live/Dead staining (green: live cells, red: dead cells) of spheroids formed with MSCs: Saos-2 cells, treated with a drug concentration of 100 nM, (C) with the presence of Nanoshuttle only in MSCs and (D) with the presence of NanoShuttle only in Saos-2, at days 2 and 7 of formation. (Scale bars 100 μ m). Viability of spheroids with the presence of Nanoshuttle only in MSCs and with the presence of Nanoshuttle only in Saos-2 cells, (E) in a drug concentration of 50 nM and (F) in a drug concentration of 100 nM. Staining with Ki-67 marker (green: cells marked with Ki-67) of spheroids formed with MSCs: Saos-2 cells, (G) with the presence of Nanoshuttle only in MSCs and (H) with the presence of NanoShuttle only in Saos-2, at days 3 and 7 of formation. (Scale bars 100 μ m). (N=6). Symbols denote statistical differences, ****p<0.0001. There are significant differences between the cell concentrations conditions. (not represented in the image for simplification). MP means magnetic particles.

In the Live/Dead assay (Fig. 18A-D), both conditions showed increased cell death by day 7, which is expected due to prolonged drug exposure. This aligns with findings by Rolder et al.[132], who reported a significant drop in viability in DOX-treated spheroids by day 7. Similarly, the CellTiter assay of both conditions (Fig.18E and 18F), demonstrated a marked decrease in viability from day 2 to day 7. In this test, on day 3, spheroids stimulated by the magnetic field exhibited greater cell death than the unstimulated spheroids, likely because DOX is more effective against proliferating cells, and magnetic stimulation promotes increase proliferation [146]. Therefore, these spheroids have a greater number of dividing cells, and the drug is more effective.

Additionally, Ki-67 staining was performed only for the 50 nM condition, revealing higher expression in spheroids exposed to magnetic stimulation (Fig.18G and 18H). Despite a slight increase in the marker in the spheroids of the condition where the NanoShuttle was present in the Saos-2 cells, the difference was minimal.

These results highlight the relevance of the 3D tumor models herein proposed, for investigating drug penetration in the context of increased tumor rigidity, a phenomenon often associated with excessive ECM production. In several cancer types, including breast, pancreatic or osteosarcoma, an enrichment of the ECM is observed, with exaggerated deposition of collagen, fibronectin and other matrix proteins. This remodeling significantly increases tumor stiffness, which not only promotes more aggressive and invasive phenotypes but also creates a physical barrier to drug diffusion.

Magnetic field stimulated spheroids represent a powerful tool to mimic this tumor microenvironment, as the stimulation promotes ECM compaction and organization. These models are therefore highly relevant for studying the relation between tumor rigidity, and therapeutic resistance. In addition, they allow the evaluation of strategies aimed at ECM modulation, such as enzymatic degradation or the use of ECM-targeting agents, to enhance drug delivery and efficacy.

Understanding these mechanisms may pave the way for combinatory therapeutic strategies incorporating ECM inhibitors to reverse resistant phenotypes.

Future studies should further explore drug responses in more complex 3D microenvironments, focusing on how spheroids with dense extracellular matrix respond to various drugs or treatment combinations. Specially, comparing the drug's efficacy in stimulated versus non-stimulated spheroids will help elucidate resistance mechanisms associated with physical changes in the tumor microenvironment.

5. Conclusion

The main aim of this study was to develop magnetic OS spheroids with the incorporation of magnetic particles. This system allows for the stimulation of ECM modification using external magnetic fields. This unique technique aims to elucidate the link between the mechanical properties of the ECM and tumor biology, thus facilitating the development of more effective treatment regimes.

The results of this work demonstrate the successful formation of compact and viable magnetic spheroids, highlighting their potential as a robust 3D tumor model. The observed increase in ECM production following stimulation with an external magnetic field suggests that this model closely replicates key features of the tumor microenvironment. Moreover, the results obtained show that the spheroids incorporated into a 3D matrix, after magnetic stimulation, lead to an increase in cell invasion and proliferation. Finally, exposure to DOX revealed that magnetic spheroids exhibited increased drug sensitivity, which can be attributed to the higher proportion of proliferating cells induced by magnetic stimulation.

In conclusion, this study demonstrates that magnetic spheroids represent a promising 3D tumor model. Future research could focus on optimizing the frequency, intensity and duration of the magnetic field to maximize its beneficial effects on spheroid viability, proliferation and pharmacological sensitivity. Furthermore, the ability to modulate extracellular matrix stiffness through magnetic stimulation provides a valuable tool to investigate how tumor rigidity affects drug penetration. Increased ECM density, as observed in our magnetically stimulated spheroids, can act as physical barrier reducing the diffusion and efficacy of chemotherapeutic agents. Conversely, understanding these mechanical changes could help design strategies to improve drug delivery by targeting ECM remodeling or optimizing drug formulations for better penetration in rigid tumor environments. This model, therefore, offers a promising platform to stimulate and study how alterations in tumor biomechanics influence treatment response, potentially guiding the development of therapies that overcome resistance caused by physical tumor properties. Additionally, the developed 3D model provides an opportunity to explore synergies between magnetic stimulation and therapeutic agents, such as chemotherapy.

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