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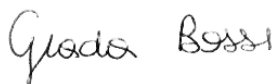
**ADVANCED THREE-DIMENSIONAL TUMOUR ENGINEERED
MODELS OF OSTEOSARCOMA AS *IN VITRO* PREDICTIVE
PRECLINICAL PLATFORMS**

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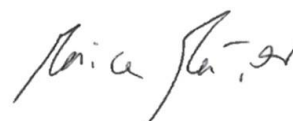


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ABSTRACT

The malignancy of Osteosarcoma (OS) is due to its high heterogeneity in terms of histological subtypes¹, subclones of tumour cells, and Tumor Microenvironment (TME)², comprising different cell types (i.e. immune cells, Mesenchymal Stem Cells - MSCs, Cancer-Associated Fibroblasts - CAFs), molecular components (e.g. growth factors and cytokines), blood vessels, environmental conditions (i.e. hypoxia, acidosis) and an aberrant extracellular matrix (ECM)³. Among tumour cells, the presence of a small percentage of Cancer Stem Cells (CSCs) with critical self-renewing and pluripotency capability is well-known to be responsible for tumour origin, development and progression as well as for relapses and metastasis. Mesenchymal Stem Cells (MSCs) have a key role in the TME as they may be at the origin of the tumour as well as involved in the tumour progression through the acquisition of a tumoral phenotype and the differentiation in Cancer Associated Fibroblasts (CAFs).

It is well-recognized the poor capability of the standard two-dimensional (2D) cell cultures in predicting the complexity of the *in vivo* tumour, providing limited reliable data for the development of potential effective therapies. A more comprehensive investigation of OS by exploiting three-dimensional (3D) platforms involving the presence of an ECM-like scaffold and smart strategies to overcome the limitations of standard cell culturing remains unexplored. The scientific community is actively working to overcome these limitations by engineering *in vitro* 3D models of OS that more faithfully replicate the tumour, to be used as predictive platforms for oncologic research and screening applications.

In this context, the design, development and investigation of two different 3D OS models by exploiting the hybrid MgHA/Coll scaffold as 3D bone-like matrix is reported in this thesis: (1) a model recapitulating the CSCs niche composed of enriched-CSCs as sarcospheres and the MgHA/Coll scaffold (3D CSCs niche model); (2) a model resembling the cellular heterogeneity of the OS and composed of osteosarcoma cells and MSCs, grown on the MgHA/Coll scaffold (3D Co-culture model).

The 3D CSCs niche model has been optimized in two stages: (1) the *in vitro* characterization of serial generations of enriched-CSCs as sarcospheres, and (2) the development of a new injection-based approach for the seeding of the sarcospheres in the MgHA/Coll scaffold, in order to create a close niche-like microenvironment. The careful selection of the sarcospheres showing the highest stem and tumoral properties, together with the optimization of a custom-based injection approach allow the development of a 3D CSCs niche model with high stem and hypoxic conditions as well as tumorigenic properties *in vivo* in Chorioallantoic Membrane (CAM) model of chick embryo.

The validation of the model in terms of drug screening application is ongoing *in vitro* and *in vivo* CAM model to investigate the potential of the 3D CSC niche model in inducing the triggering of cellular mechanisms commonly activated during the drug resistance phenomenon to different chemotherapeutics.

The 3D co-culture system was developed by seeding GFP-MNNG HOS osteosarcoma cells and MSCs at 1:1 ratio on the MgHA/Coll scaffold and the interaction between the two cell types and the ECM has been investigated. The 3D Co-culture models exhibited favorable cell behavior and suggested an alteration of the mesenchymal phenotype and MMPs production, confirming the role of the cell-cell and cell-ECM interactions in the TME and the importance of improving the complexity level of the cell culture systems to unravel pathological pathways.

In conclusion, both the 3D models of OS proposed offer promising prospects for the development of sophisticated systems to explore the biology of the malignancy as well as for the screening on new therapeutic approach.

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1. INTRODUCTION

1.1. The Osteosarcoma

1.1.1. *Epidemiology, aetiology, clinical manifestation and diagnosis*

Osteosarcoma (OS) is the most common primary solid malignancy of bone sarcomas, with an overall incidence of two-thirds of primary bone tumours. OS incidence reaches about 2-3/million/year, with an annual peak in adolescence at 15-19 years of age⁴ and with males affected 1.4 times more frequently than females⁵. A second peak of OS in adults over 65 years of age has been reported, but it has been demonstrated to represent a second malignancy related to a main disease⁶. Approximately 80% of patients with OS present a localized primary tumour, while the other 20% present initial pulmonary metastases⁷. For metastatic patients, the overall survival rate is less than 20%⁷. Mostly, the overall survival of recurrent OS patients is lower than 28%⁸; indeed, OS recurrence is possible a long period (2-3 years) after the patient completes the treatment, during which he is found to be cancer-free⁴.

The 80% of OS occurs in the metaphysis of extremities, especially of long bones and distal femur, where the proximal tibia and humerus are the most recurring primary sites, with 50% originating around the knee⁹.

The sites of maximum growth together with the OS predilection for the puberty age suggest a correlation with rapid bone proliferation. On the other side, a minority of OS is caused by irradiation or alkylating agent exposure. It was also demonstrated an increase in OS incidence in the presence of well-defined hereditary disorders associated with germline alterations of tumour suppressor genes, such as hereditary retinoblastoma⁴ and the Li-Fraumeni cancer family syndrome¹⁰.

Local pain, followed by localized swelling and the identification of a palpable mass at the site of the pain are the typical clinical manifestations and symptoms of OS⁷. A pathological fracture can be the first sign of disease and radiographic metastases in the lung, bone and lymph nodes can be observed in approximately 15% of patients⁹.

The diagnosis of OS involves the full story of the patient, physical examination and plain X-ray radiographs to observe the tumour localization and the whole extremities¹¹. OS is characterized by a soft tissue component where patchy calcifications derived from new bone formation can be observed. Specifically, a triangular area of periosteal calcification in the border region of the tumour and healthy tissue, known as the Codman triangle, is typically observed in OS¹². Magnetic resonance imaging (MRI) is the best modality to assess these osseous changes and their relationship with the surrounding tissues, vessels and nerves, as well as their intramedullary extension which are essential for safe

surgery¹². Potential metastatic sites are evaluated by a CT (Computed Tomography) scan of the thorax and a ^{99m}Tc bone scan of the whole skeleton⁴.

OS lacks specific markers, but an increased level of lactate dehydrogenase (LDH) or alkaline phosphatase (ALP) in serum are usually detected in patients, both associated with lower outcomes⁴. The diagnosis of OS is also verified histologically mostly by open biopsy. The hallmark of diagnosis is the presence of proliferating malignant Mesenchymal Stem Cells (MSCs) together with the production of osteoid and/or bone, chondroid and fibrous matrix by these tumour cells, reflecting the mesenchymal origin of the malignant cells. The three major subtypes of conventional OS, osteoblastic, chondroblastic and fibroblastic, are classified depending on the predominant type of matrix within the tumour⁴. Moreover, additional histological variants, including small cell OS, parosteal and periosteal OS, low-grade central and high-grade surface OS, are recognised by the scientific community⁴.

1.1.2. Cell-of-origin, tumour development and progression

The cell-of-origin concept refers to the normal cell type that acquires the first cancer-promoting mutations and initiates tumour formation and several categories of cancers (i.e., carcinomas, sarcomas, myelomas etc.) are identified depending on the cell type of origin¹³.

OS is a sarcoma, which means that it has a mesenchymal cell of origin. Indeed, the osteosarcomagenesis typically occurs from a single MSC located in the bone marrow, which, once committed to the osteogenic lineage, will give rise to a polyclonal, heterogeneous tumour mass due to p53 and Rb tumour suppressor excision¹⁴. Indeed, OS initiates as a monoclonal disease, which quickly develops into a heterogeneous polyclonal disease comprised of an array of distinguishable sub-clones, making it the most complex cancer in terms of molecular aberration¹⁵.

More recent data and the vast heterogeneity of OS suggest that it can form at multiple points during bone development, from both MSCs and osteoblasts as well as osteoclasts¹⁰, and that no single driver gene can be pinpointed to be the cause of osteosarcomagenesis as several signalling pathways cause genetic instability in OS development¹⁵. Indeed, the peak incidence of OS during the time of the puberty suggests a connection between intensive bone growth and OS¹⁶. It has been demonstrated that a change in cell cycle regulators can occur in osteoblast precursors, orchestrated by signals from the tumour microenvironment and responses to genetic and epigenetic changes within these cells¹⁷. Genes related to osteoblast differentiation from MSCs have been associated with osteosarcomagenesis, including the aberrant activation of Wnt protein family members¹⁸, the overexpression of Bone Morphogenetic Proteins (BMPs)/Transforming Growth Factor (TGF) family members¹⁹ and Runt-related Transcription Factor 2 (RUNX-2)²⁰ and elevated Alkaline Phosphatase (ALP) serum levels²¹. Moreover, OS can be osteolytic, suggesting an increased osteoclast activity

whose regulation depends on cytokines and growth factors, such as insulin-like growth factors (IGFs), Receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG)²².

Besides the multiple pathways and cells involved, the osteosarcomagenesis is still not fully explained. In the recent years, a new theory has been complemented regarding the origin of OS occurring from Cancer Stem Cells (CSCs). In this theoretical model, the OS is thought to be hierarchically organised with a subpopulation of self-renewing cells, namely CSCs, at the basis of tumour development and progression²³. According to this theory, CSCs arise either by transformation of MSCs, or by transformation of progenitor cells of the osteoblastic lineage following changes in cell cycle regulators²⁴.

The increased migration and invasion ability of OS cells promoting the tumour progression and metastatic process may be related to different pathways. Among the most relevant, Matrix Metalloproteinase (MMPs) are enzymes responsible for the degradation of extracellular matrix (ECM) proteins during normal remodelling, but the overexpression of MMP-2 and MMP-9 in the OS assumes a promotional role for metastatic process²². It is widely recognized the critical role of vesicles as intercellular communication mediators; it was found that MSCs can internalized tumoral vesicles, assuming the role of “tumour-directed MSCs” which promote OS growth and metastasis²⁵.

1.1.3. The Heterogeneity of the Osteosarcoma

According to the World Health Organization (WHO), “OS is an osteogenic tumour that produces defective bone with osteoid deposition or bony matrix”²⁶. In addition to the different OS subtypes depending on the predominant type of ECM²⁷, in a single OS, a heterogeneous set of tumour cells that differ in terms of morphology, phenotype, gene expression, metabolism, immunogenicity and proliferative and metastatic potential is presented. Among them, CSCs represent a unique subset of self-renewing clones that play a key role in achieving heterogeneity by constantly enriching the tumour mass with new mutated cells and dominant subclones and by regulating the Tumour Microenvironment (TME)²⁸. Indeed, the concept of TME has been appreciated in recent years based on increasing evidence showing that the presence of the tumour depends on the strict cooperation among tumour cells within it and the components of the surrounding TME, creating a supportive niche with heterotypic signalling that favour cancer cell survival, outgrowth and escape from the immune system²⁹. It is possible to describe the TME as a dynamic niche composed of a set of cellular (immune cells, MSCs, cancer-associated fibroblasts – CAFs, epithelial cells) and molecular components (growth factors and cytokines), capillaries and the ECM³, that closely interact with cancer cells to meet their nutrient and metabolic demands³⁰.

1.1.3.1.CSCs

Gibbs et al. firstly prove the existence of CSCs of OS by identifying a subpopulation of cells that was able to form spheres, named sarcospheres, in serum free and anchorage-independent conditions in OS tissue samples³¹. CSC is commonly defined as a tumour cell with stem cell-like properties. Similar to normal stem cell, CSCs has self-renewal potential, differentiation ability and can expand by symmetrical division³². Self-renewal is the inherent ability of stem cells to generate two daughter cells, in which one of them preserves the stem-cell state, namely developmental capacity; the other immature and unspecialized daughter cell can undergo differentiation acquiring new individual properties and functions²⁴. These properties make CSCs responsible for tumour formation³³, metastasis³⁴, chemotherapy resistance³⁵ and heterogeneity, as they differ genetically and phenotypically among OSs³⁶.

The CSCs express specific surface markers associated with stemness and/or tumorigenesis, such as CD133 (Prominin-1), CD117 (pro-oncogene c-Kit) and CD127 (Interleukin-7 receptor), commonly used to sort positive cells from OS cell lines. Some pluripotency-associated markers, such as OCT-4 (Octamer-binding transcription factor 4), SOX-2 (Sex determining region Y-box 2) and NANOG (Homeobox transcription factor Nanog) are expressed by CSCs.

CSCs are characterized by drug resistance thanks to the activation of specific functions such as a high DNA repair mechanism, enhanced protection against reactive oxygen species (ROS)³⁷, the alteration of ATP-binding cassette family of efflux transporters (ABC transporters) (e.g., ABCG2 and ABCB1)³⁸ and a high expression of ALDH-1 (aldehyde dehydrogenase 1) which is associated to chemotherapy and metastatic potential³⁹.

The presence of CSCs defines the TME in which they reside, defined as niche, and the TME in turn influences the CSCs properties, modulating their entry into a state of rest, retention of stemness features and providing them physical support⁴⁰. In this regard, signals of the bone microenvironment, including those mediated by fibroblastic growth factor (FGF), transforming growth factor β (TGF- β), IGF-1, BMPs, hypoxia-inducible factors 1 alpha (HIF-1 α) and Notch homolog 1 translocation-associated (NOTCH-1) are deregulated in OS and the literature reports their involvement in the regulation of the self-renewal, differentiation, growth, drug resistance and/or metastatic potential of CSCs⁴¹. Three niches within the OS where these signalling pathways are particularly active could be inferred to be the location of CSCs; (1) the perivascular niche, (2) the hypoxic niche and (3) the endosteal niche. Indeed, the perivascular location favours the migration and metastasis of CSC⁴², while the hypoxia-induced signalling is a key environmental factor involved in the stemness preservation, OS progression and drug resistance of CSC⁴³. The endosteal niche is a rich environment where tumour cells interfere with the bone remodelling process, establishing a “vicious cycle” that

favours osteoclast-mediated osteolysis and the subsequent growth factors' release which support stem and tumoral properties⁴⁴.

1.1.3.2.MSCs and CAFs

The MSCs are non-hematopoietic precursor cells found in numerous tissues (e.g., bone-marrow, peripheral blood, umbilical cord, placenta, adipose tissue) that have the self-renewal ability and pluripotent differentiation potential into various mesenchymal cell lines, such as osteocytes, chondrocytes, myocytes and adipocytes with the final aim to maintain body homeostasis⁴⁵.

However, MSCs assume a critical role in the OS; the OS development and progression may be accelerated by two types of MSCs, namely normal tissue MSCs and tumour tissue MSCs⁴⁶. Normal MSCs can be recruited from a distant site to the TME by cancer cells by releasing cytokines (e.g., Interleukin 6 – IL-6 and platelet-derived growth factor – PDGF) and “educated” into heterogeneous pro-tumoral phenotypes⁴⁶. On the other side, tumoral MSCs dramatically accelerate the proliferation and metastasis of OS cells. For example, a synergistic cross-destruction circuit it's present between tumoral MSCs and OS cells; indeed, OS cells interact with tumoral MSCs in the TME through the release of Interleukin 8 (IL-8), which in turn stimulates the expression of IL-8 in tumoral MSCs that, is received by OS cells accelerating their expression of IL-8⁴⁷, creating a continuous circuit which increases the invasiveness and self-renewing properties of tumour cells⁴⁸.

Moreover, various mechanisms are involved in the induction of the stem-like phenotype of tumour cells and the restoration of CSCs through MSCs interaction, (1) including cell fusion, (2) direct transformation of MSCs into CSCs, (3) the crosstalk between MSCs and CSCs/tumour cells mediated by the secretion of growth factors and cytokines⁴⁹. (1) The cell fusion phenomenon involves the fusion of stromal cells with tumour cells and different studies demonstrated the presence of hybrid cells with metastatic, tumorigenic and stem-like properties^{50, 51}. The cell fusion leads to nuclear reprogramming and it may clarify the origin of CSCs and the mechanism for cancer metastasis and carcinogenesis⁵². (2) MSCs can also be directly transformed into CSCs; indeed, it has been demonstrated that the methylation of tumour suppressor genes (e.g. HIC1 and RasF1A) in MSCs can lead to the transformation of stromal cells into CSCs, increasing the overall drug resistance and recurrence ability of the tumour⁵³. MSCs also can reprogram toward CSCs following aberrant changes in the TME, through the increased production of OCT-4, SOX-2 and NANOG as well as the activation of Wnt and Hedgehog signalling pathways^{54, 55}. (3) The crosstalk between MSCs and CSCs/tumour cells is highly complex. MSCs release TGF- β which can stimulate the NOTCH-1 (Notch homolog 1, translocation-associated) signalling pathway contributing to the preservation of the CSCs phenotype⁵⁶ and, together with tumour necrosis factor α (TNF- α), to facilitate the tumour immune escape⁵⁷. The IL-6 has a key role in OS as it secreted by both MSCs and tumour cells^{58, 59}.

and it increases the drug resistance and stem-like phenotype of both cell types, acting as a direct regulator of CSC self-renewal and creating a positive communication feedback loop⁵⁹. Moreover, it has been reported that IL-6, together with VEGF (Vascular Endothelial Growth Factor), promote tumour angiogenesis and growth when MSCs are co-culture with OS cells⁵⁸. Emerging evidence reported also that the VEGF by MSCs may promote metastasis increasing the migration ability of OS cells⁶⁰.

MSCs contribute to OS also through their differentiation into CAFs under the education of tumour cells. CAFs commonly originate from tissue-resident fibroblasts activated by the TME⁶¹, however, also smooth muscle cells, endothelial cells, adipocytes, pericytes, epithelial cells and bone-marrow mesenchymal stem cells (BM-MSCs) can give rise to CAFs⁶² creating a heterogeneous population with different fibroblast-like phenotypes and functions in many cancer types⁶³. In addition, it has been demonstrated a different degree of CAFs activation which can generate different CAFs subtypes in the same tumour playing a variable role in the tumour progression⁶⁴. For this reason, a universal definition and exclusive markers for CAFs have not been identified yet. Generally, CAFs can be stated as the mesenchymal fibroblastic component of TME⁶⁵ that, as normal fibroblasts, should be negative for epithelial (EpCAM), endothelial (CD31) and leukocyte (CD45) markers and have an elongated spindle-like morphology⁶⁶. More specific markers, such as fibroblasts activation protein (FAP) and α -smooth muscle actin (α -SMA), have been recently identified to help to distinguish CAFs from normal fibroblasts⁶⁵.

The differentiation of BM-MSCs into CAFs is a multistep biological process which involves an epithelial-mesenchymal transition, environmental conditions, cellular communication and cytokines⁶⁷. It has been demonstrated that BM-MSCs can be transformed into CAFs via contact with OS cells, through the increased expression of IL-6 and IL-8 in the TME⁶⁸. Another study showed that OS cells can induce BM-MSCs differentiation into CAFs with the promotional role of NOTCH and Akt signalling pathways⁶⁹.

One of the CAF's functions is the pro-stemness ability as a specific subtype has been identified as proficient in secreting pro-stemness paracrine factors^{70,71} which support CSCs properties and increase the transformation of tumour cells into CSCs. In addition, after exposure to chemotherapeutics, CAFs are stimulated to produce even more pro-stemness cytokines and chemokines that increase the tumour aggressiveness following cancer treatment⁷². Moreover, CAFs interact with tumour cells by secreting proteins and cytokines such as TGF- β , IL-6 and IGF, regulating immune feedback and the ECM remodelling⁶³.

1.1.3.3.Extracellular matrix (ECM) and environmental conditions

In the TME, dynamic processes such as the ECM aberrant remodelling and signalling, variation of oxygen and nutrients, and mechanical interactions typically occur and reciprocally affect its multiple cellular and molecular components⁷³.

The ECM is a three-dimensional acellular network of macromolecules which provide the necessary structural and biochemical support to its cellular components⁷⁴, regulating most cellular behaviours (e.g., communication, migration, adhesion, proliferation and differentiation)⁷⁵. However, the dysregulation and aberrant remodelling of the ECM in the TME hijacked its standard functions and a new ECM profile that enables metastatic dissemination of cancer cells is formed⁷⁶. In addition, the formation of an osteoid matrix directly produced by cancer cells acts as a supportive scaffold for rapid tumour progression²⁵. Elevated concentrations of ECM components (e.g., collagen, fibronectin, laminin and proteoglycans)⁷⁷ have been found in the OS patients and they have been demonstrated to be related to increased invasiveness⁷⁸, metastasis^{79,80} and drug resistance⁸¹. The function of the ECM is not only derived from its composition, but also from the aberrant constitution in terms of macromolecules, proteases, inhibitors and their downstream signalling pathways⁸²; MMP-2 and MMP-9 have been found to be upregulated as significant proteolytic enzymes degrading ECM and enabling OS cell invasion and metastasis⁸³.

One of the main traits of solid tumour is the intra-tumoral hypoxia due the excessive proliferative activity of cancer cells in consuming large amount of oxygen in the microenvironment, resulting in the formation of a hypoxic zone in the central area of the tumour⁸⁴. It's only when the hypoxic microenvironment reaches an oxygen concentration <5% that the expression of hypoxia-inducible factor-1 (HIF-1), specifically the hypoxia-inducible oxygen-dependent subunit HIF-1 α ⁸⁵, is stably induced. HIF-1 α provides cancer cells the ability to adapt to hypoxia often promoting their dedifferentiation into CSCs by increasing and preserving the expression of stem cell markers such as CD133, OCT-4 and NANOG⁸⁶.

Extracellular acidosis facilitates the chemotherapy or radiation resistance, invasion, neovascularization and stemness in OS⁸⁷. The imbalance between oxygen consumption by cancer cells and insufficient oxygen delivery from aberrant tumour vasculature is the main reason for such phenomenon, together with the high-rate energetic glycolytic metabolism of cancer cells⁸⁸.

It's known that under physiological conditions the organisms experience different biomechanical forces (e.g. shear stress, matrix stiffness, tension and compression pressure)^{89,90}. The tumour includes excessive cell proliferation which leads to an abnormal biomechanical environment in terms of solid stress, increased matrix stiffness and abnormal interstitial fluid pressure^{91, 92}; however, in the OS a decreased stiffness can be observed due to osteolysis. The biomechanical properties of the TME are

essential for the maintenance of the homeostasis of the CSC population as mechanical signals induce the reprogramming of tumour cells into CSCs by increasing the expression of SOX-2, OCT-4 and NANOG^{93, 94}.

1.2. Therapeutic regimen and outcomes

1.2.1. Conventional therapeutic regimen and innovative treatments

Current therapeutic regimen for localized disease involves primary pre-operative chemotherapy (neoadjuvant), followed by surgery and, then, post-operative (adjuvant) chemotherapy, sometimes accompanied by radiotherapy⁹⁵, for at least 6-8 months total duration of chemotherapy⁹⁶. The treatment for metastatic and relapsed OS is the same as that of localized disease, with the mandatory surgical removal of all known metastatic foci. The second-line chemotherapy for OS relapses is less defined than that of surgery and there is no accepted standard regime⁷.

The systemic therapy involved the simultaneous use of different chemotherapeutics, such as doxorubicin (DOX), cisplatin (CIS)⁹⁷, ifosfamide (IFO)⁹⁸ and high-dose methotrexate (MTX)⁹⁹, as most active agents against OS¹⁰⁰. The most ideal combination of agents remains to be defined. CIS is an alkylating agent which induces DNA damage through the link between platinum ions and DNA bases, inhibiting DNA replication and cell division¹⁰¹. DOX is an anthracycline which causes DNA damage by inhibiting topoisomerase II and DNA replication¹⁰². IFO acts as an alkylating agent which creates irreparable cross links between DNA strands which stops DNA replication¹⁰³. MTX targets dihydrofolate reductase (DHFR) which is a key enzyme in the folate cycle and a metabolic component of nucleotide biosynthesis¹⁰⁴.

It's well-known the toxicity of chemotherapeutic agents on patients; while CIS causes nerve damage¹⁰¹, cardiotoxicity is associated with DOX¹⁰². IFO damages the lining of the bladder¹⁰³ and MTX exhibits high rates of renal and liver toxicity, leukoencephalopathy and brain damage⁹⁹.

The scientific community is actively working to target novel pathways of interest in OS with over 500 clinical trials involved (e.g. p53 and RB genetic abnormalities¹⁰⁵, mutations in ABC family members as drug efflux pump¹⁰⁶, RANK signalling¹⁰⁷ and Tyrosine Kinases¹⁰⁸). Immunotherapies have come to the forefront in recent years as anticancer therapies¹⁰⁹ as well as the combination of metabolic therapies¹¹⁰. Recently, MSCs have been identified as possible delivery system for therapeutic agents via target-specific engineering processes¹¹¹. Similarly, drug-conjugated nanomaterials with modulated protein corona composition can be used to have endogenously triggered selectivity¹¹². Again, selenium (Se)-based organic compounds have recently attracted a growing interest as potential anti-cancer agents¹¹³; multiple works reported their high efficiency and selectivity against cancer cells due to the Se pro-oxidant effect that alters cellular redox homeostasis. The real anti-neoplastic properties are still unclear, but it is accepted that Se compounds

mechanistically impair mitochondrial structure and function liberating organelle-buffered iron to inhibit tumour cells by ferroptosis. Organic Se compounds specifically show a significant anti-tumoral activity with a reduction of metastasis and fewer system side effects compared to inorganic compounds¹¹⁴.

1.2.2. The Drug Resistance Phenomenon

Besides the contributing role of TME and its components in drug resistance⁸¹, cancer cells exploit various specific molecular mechanisms to circumvent the cytotoxic stimuli induced by therapies.

Among them, the impairment of drug transport as a reduction in transporters on the membrane of tumour cells, typically used for MTX resistance response, and the enhancement of drug efflux pumps for DOX resistance, are the most common mechanisms. In the second case, the overexpression of members of ABC transporters on OS cells (i.e., multidrug resistance protein 1 – MDR1, ABC subfamily B member 1 - ABCB1) is the distinctive sign.

Alterations in enzymes can both increase the levels of the target enzyme or decrease drug affinity, resulting in drug resistance; this is the example of MTX-resistant OS cells which overexpressed dihydrofolate reductase - DHFR^{115, 116}, while in DOX-resistance, DNA topoisomerases II (TOP2), as nuclear enzymes involved in the regulation of DNA topology, is downregulated in OS cells¹¹⁷.

It is well known that DNA damage is the principal action mechanism of chemotherapy³⁷. OS cells can occasionally increase their DNA repair mechanisms in response to the DNA damage caused by chemotherapeutics. For example, OS cells respond to drugs by involving the Nucleotide Excision Repair (NER) family which can repair the chemical drug-induced damage¹¹⁸; among NER members, the overexpression of excision repair cross-complement proteins (ERCC) which excise the lesion followed by DNA replication and repair process is involved in CIS resistance¹¹⁹. Base Excision Repair (BER) family repair DNA damage caused by small chemical alterations or base loss due to the hydrolysis of glycosylic DNA bonds¹²⁰.

Alterations in the cell cycle and cell death mechanisms (e.g., apoptosis and autophagy) are also presented. OS cells can arrest their cell cycle to repair DNA damage evading apoptosis¹²¹. Cyclin-dependent kinase 4 (CDK4) is an enzyme responsible for the phosphorylation of the RB protein during the G1-S phase that is highly expressed in OS cells¹²², compared to normal osteoblasts, and it's associated with resistance to CIS¹²³. The members of the B cell lymphoma 2 family (BCL-2) both pro- and anti-apoptotic are typically altered in OS¹²⁴; among them, Myeloid cell leukemia-1 (MCL-1) as a pro-survival member, is overexpressed in OS and associated with decreased sensitivity to MTX⁸¹. Autophagy is also an important strategy for malignant tumour to promote survival and induce resistance to chemotherapy together with its role in the maintenance of CSCs stemness¹²⁵.

Various signal transduction pathways are activated during drug resistance such as human epidermal growth factor receptor 2 (HER2) which is overexpressed in 32%-45% of OS samples and associated with poor response to CIS¹²⁶.

The miRNAs are small non-coding RNAs that have been demonstrated to be involved in various processes of the OS, including drug resistance¹²⁷.

1.2.3. The Drug Discovery and Development Process

The Drug Discovery and Development Process (DDDP) is the costly process that takes a new potential drug from the laboratories to market approval and post-marketing surveillance¹²⁸. The journey of discovering a new drug is estimated to cost around \$2.6 billion, without considering the post-marketing monitoring and development costs reaching a total of about \$3 billion for each new drug. Moreover, the process faces increasing costs and challenges, that overall lead to the discarding of most of the drug candidates and to get just a few compounds to the market¹²⁸.

The 1° stage of the process is called “early drug discovery” concerning basic research for the identification of a macromolecule, a dysfunctional signalling pathway or a molecular mechanism linked to a disease condition. Scientists attempt to identify the putative therapeutic target and to study its mechanisms of interaction with other, unintended targets, defining the hypothetical therapeutic agent that modify the target’s function and restore health or alleviate symptoms¹²⁹. The poor knowledge about the mechanisms behind the origin and progression of a disease is particularly true for the OS, thus a better understanding of the tumour may help to perform more comprehensive assays using emerging tools and technologies to identify potential biomarkers that can be used as targets; this, in turn, may lead to the development of more targeted therapeutics¹³⁰.

The 2° stage concerns the identification of the therapeutic agents modulating the selected target. Once they are identified, the preclinical phase (3° stage) starts to test the agents *in vitro* and a few promising molecules are then evaluated *in silico* and *in vivo* models of human disease, with the aim to provide sufficient evidence of safety and efficacy before clinical trials in humans¹²⁸. Thousands of potential drugs are tested *in vitro*, however, the transition to *in vivo* preclinical studies represents a bottleneck of the DDDP as a huge number of drugs fail to show safety or any therapeutic effect. This is due to the *in vitro* use of bi-dimensional (2D) models in drug screening that are not sufficiently predictive of the *in vivo* microenvironment; these models are simple, economical and quick platforms concerning the culture of cells on plastic flat surfaces (e.g., petri dishes and flasks) in an unrealistic environment with an oversupply of nutrients and gases and the absence of a tissue-like ECM¹³¹. On the other side, animals and humans are three-dimensional (3D) environments, where different cell types are strictly connected to the surrounding composed of ECM and blood vessels, and where the time, absorption, distribution and elimination way of the potential drug in the body can be evaluated.

After the approval of the Investigational New Drug (IND) application by regulatory agencies (e.g., the European Medicines Agency – EMA in the EU) reporting preclinical efficacy data and toxicity, the manufacturing information and the clinical protocol proposed for the clinic, three phases (Phase I, II and III) of clinical trials start (4° stage) to test the drug candidates in humans¹³². In Phase I, the safety and tolerability of the agent are tested in a small number of healthy individuals (20-80 people). In Phase II, 100-500 patients are used to test if the therapeutic agent provides the desired effect, while in Phase III, the efficacy of the therapeutic agent is evaluated in a larger patient population (1000-5000 patients) relative to the standard of care or a placebo, and possible interactions with other medications¹³². However, a big bottleneck affects this stage due to the inconclusive data of most *in vivo* preclinical studies. The passage from the *in vivo* preclinical phase to clinical trials leads to a potential mismatch of preclinical and clinical endpoints in terms of failure of efficacy and human toxicity by drug candidates not previously shown in animal studies. The mismatch is due to the limitations of animal models in developing all the symptoms and pathological characteristics of the disease, in predicting human responses and in potentially forming abnormal phenotypes. It is estimated that less than 14% of drug candidates entering phase I of clinical trials can gain agencies approval¹²⁸. A New Drug Application (NDA) is submitted to the regulatory agencies to decide for market approval (5° stage). The approval is always followed by a post-market monitoring phase, called “pharmacovigilance” during which the drug is observed with infinite duration to identify the onset of potential delayed side effects or caused by long-term administration¹³².

It's clear that these bottlenecks lead to a waste of time, money and resources and innovative solutions need to be found to reduce the translational failure of drug candidates in the process. Moreover, the process involves many disciplines, it implies the generation and use of a vast amount of data obtained via different types of high-throughput technologies and it's very long, expensive and high-risk. Indeed, the pre-discovery stage (1° and 2° stages) lasts about 5-6 years, followed by 2-3 years of preclinical phase (3° stage), 4-7 years of clinical trials (4° stage) and 1-2 years for review and approval (5° stage)¹³².

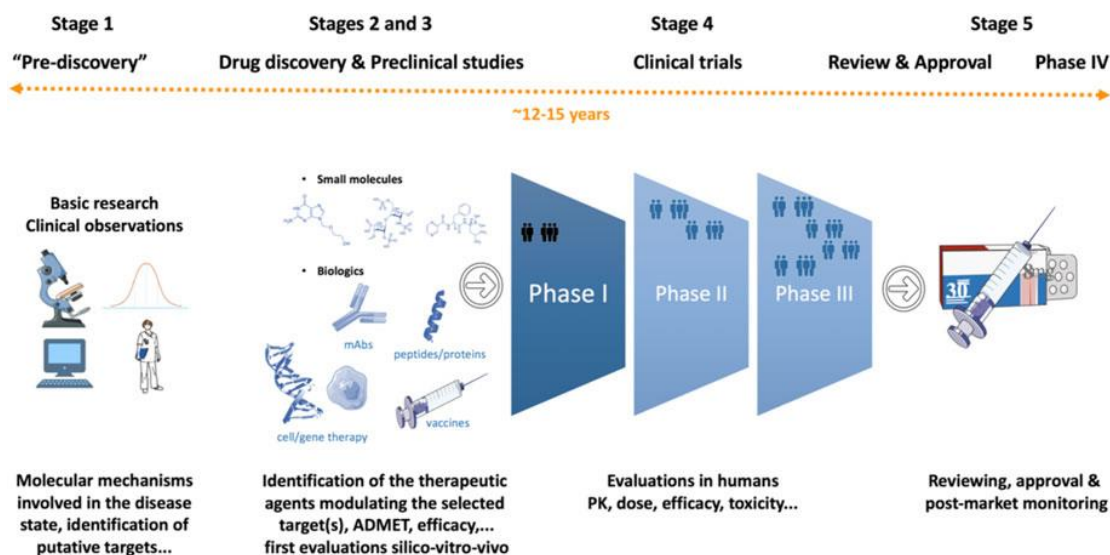


Figure 1. Drug Discovery and Development Process. The stages of the process are represented in a highly simplified manner. The figure has been taken from¹³³.

1.3. *In vitro* Tissue Engineering evolution: Tumour Engineering

1.3.1. *The third dimension (3D): from tissue to tumour engineering applications*

The scientific community is actively working to engineer *in vitro* 3D models that can closely reproduce human tissues as predictive platforms for *in vitro* testing, with the final aim to (1) reduce the gap between *in vitro* and *in vivo* preclinical studies, (2) capture the *in vivo* microenvironment in a laboratory setting, (3) improve tests outcomes and, eventually, (4) reduce the use of animal models when possible. Significant efforts have been made to reproduce in the lab the dynamic interaction between cellular components and the TME, leading to the Tissue Engineering's evolution into "Tumour Engineering", whose aim is engineering the three-dimensionality of the tumour *in vitro*, elucidate pathological events and discover new therapies¹³⁴. Several technologies of Tissue Engineering field are currently available to develop 3D *in vitro* models, such as (1) multicellular spheroids and organoids, (2) biomaterials, and (3) microfluidic devices¹³⁵; however, these models are still unripe and need a deep optimization to be performed to reach the complexity of the *in vivo* tissues.

1.3.2. *Multicellular spheroids and organoids*

Multicellular spheroids are cellular aggregates growing as spheres derived from the intrinsic self-assembly of cells in suspension. They better reproduce the *in vivo* cellular networks that affect their functionality, viability, polarity, protein secretion and gas/nutrient exposition¹³⁶. The spheroid is characterized by a limited diffusion of biomolecules and gases due to the formation of a compactly organized aggregate of cells creating a decreasing gradient of nutrients and oxygen from the outer layer to the core of the spheroid, giving rise to a layered structure composed of a highly hypoxic necrotic core surrounded by a quiescent zone and a proliferating outer layer¹³⁷.

The spheroids are used to study cell metabolism, tumour biology, and toxicology and to engineer *in vitro* microtissues due to their high reproducibility and low related-costs¹³⁸. On the other side, spheroids show variable size, poor control on cell viability, functions and differentiation within the spheroid and, mostly, the absence of an ECM¹³⁹.

Different techniques can be used to produce spheroids, such as non-adherent surfaces, magnetic levitation, force-floating methods and specific microfabricated platforms¹³⁹.

The use of non-adherent surfaces is the most cost-effective and easy method for spheroid production and it consists on the cell growing in a non-adhesive substrate which promotes cellular aggregation while prevents cellular adhesion to the substrate¹⁴⁰. Recently, a revised version of this method has gained popularity to be used on tumour cells to enrich a subpopulation of stem-like cells, namely CSCs, that can grow as anchorage-independent free-floating spheres³¹ in a wide variety of tumours, including OS, brain, breast and head and neck squamous cell carcinoma^{8, 141, 142}. This protocol, called “sphere-forming culture” guarantees the partial enrichment of CSCs by combining the non-adhesive substrate with the absence of serum supplementation in the culturing media, well-known for its differentiation effect¹⁴³, and the addition of growth factors (e.g., Endothelial Growth Factor – EGF, basic Fibroblast Growth Factor – bFGF, N2 and B27) as supporters of CSCs proliferation, faster diameter increasing and the overexpression of stemness markers (i.e., CD44, CD133, SOX-2, OCT-4, NANOG¹⁴⁴). However, the absence of a relevant ECM is still a limitation that need to be considered for the investigation of the tumour microenvironment¹⁴⁵.

The organoids are miniaturized organs with a 3D structure composed of multiple cell layers of tissue-specific cell types and stem cells within a unique intrinsic organization¹⁴⁶. Organoids can be used to model diseases, such as infectious diseases of the gut¹⁴⁷, stomach¹⁴⁸, neurodegenerative disorders¹⁴⁹ and cystic fibrosis¹⁵⁰. However, organoids have different drawbacks, such as their small size, the lack of vascularization, the absence of tissue-specific cell densities, the inadequate control of nutrient supply, the improper removal of cellular waste products, the insufficient exchange of gases and nutrients and poor reproducibility¹⁵¹.

1.3.3. Biomaterials

Naturally derived or synthetic matrices, scaffolds, hydrogels and nanostructured systems have been used as suitable platforms for the *in vitro* support of cell adhesion and proliferation and, mostly, as tissue-like ECMs mimicking the native tissues¹⁵². Electrospinning¹⁵³, freeze-casting¹⁵⁴, 3D printing, bioprinting¹⁵⁵ and decellularization of tissue¹⁵⁶ represent the most frequently used manufacturing techniques for the fabrication of these supports.

The decellularized matrix (dECM) is obtained through the removal of cellular and nuclear components from the native tissue by treatments with different chemical, biological or physical agents

to obtain natural-derived matrices which preserve the structure, ECM components and growth factors of the original one¹⁵⁷. The synthesis of a liver-derived scaffold¹⁵⁸, cartilage¹⁵⁹, pancreas¹⁶⁰, bone¹⁶¹, spinal cord¹⁶², kidney¹⁶³ and also cancers¹⁶⁴ has been reported with this approach. The dECM can also be used as bioink for printing/bioprinting highly mimetic hydrogels compared to those obtained from commercial inks¹⁶⁵. Different organs such as lung¹⁶⁶, kidney¹⁶⁷, brain¹⁶⁸, spinal cord¹⁶⁹, bone¹⁷⁰, colon¹⁷¹ and umbilical cord¹⁷² have been successfully printed. However, a challenging scaling up and standardization of the process still affect this approach¹⁷³.

Electrospinning technology processes solutions or melts of polymers into homogenous fibres with diameter distribution from nanometres to sub-micrometres using a high electric field¹⁷⁴. These scaffolds have highly porous structures with large surface-to-volume ratios that support cell interactions and adhesion, making them attractive for tissue engineering and modelling applications. Electrospinning offers the possibility of large-scale production, easy functionalization and material combinations, but the chaotic sub-microfibre deposition could form a tightly packed network whose pores are too small for cell infiltration¹⁷⁵.

The freeze-casting method, known also as ice templating, is a versatile technique to tailor pore structure and mechanical properties by manipulation of parameters such as the degree of porosity, pore size, pore shape and orientation during scaffold fabrication according to tissue requirements¹⁷⁶. Different works reported successful porous scaffolds for regenerative medicine and tissue engineering applications¹⁷⁷⁻¹⁷⁹, however, the biocompatibility and biodegradability of freeze-casted scaffolds are the most restrictive factors¹⁸⁰.

3D printing and bioprinting represent the most promising approaches for the development of tissue-like matrices because they provide the control of the structure in all directions during the process thanks to the digital design of the frame using computer-aided design software or scanning from medical images before printing¹⁵⁵. Moreover, the 3D bio-printing allows the direct pattern of cells within the material facing the limitations of traditional cell seeding on pre-fabricated scaffold¹⁵⁵. Different works report the bioprinting of neural tissue¹⁸¹, spinal cord¹⁸² and nerves¹⁸³. However, commercially available bioprinters have a high cost and low customization capacity and they require costly consumables and a high workforce for maintenance, limiting their potential applications¹⁸⁴.

The concept of “fourth dimension” has been recently introduced concerning the use of stimuli-responsive (i.e., magnetic field, electric field, ultrasounds etc. responsive materials) smart biomaterials to easily control and manipulate the architecture, orientation or cues gradient within the scaffold¹⁸⁵. However, this is a quite new and still unripe field.

1.3.4. Microfluidics and bioreactors

Microfluidics represents the science and technology of systems that manipulate small (10^{-9} to 10^{-18} L) amounts of fluids using channels with dimensions of tens to hundreds of micrometres¹⁸⁶. Depending on their application, microfluidic devices can be used as microreactors¹⁸⁷, lab-on-a-chip¹³⁹ or organ-on-a-chip¹³⁸ and they can be manufactured from a broad range of materials¹⁸⁸ with different costs and advantages depending on their final destination use¹⁸⁷. In microfluidics, working at the microscale level, the material properties are more amplified, affecting the entire platform in terms of shorter retention times, laminar flows, enhanced heat and mass transfer and large surface-to-volume ratios¹⁸⁹.

For this reason, microfluidics has gained research interest for the production of chips with great potential in personalized medicine, disease diagnosis^{190, 191} and chemical screening, cell culture, drug screening and drug delivery, and DNA sequencing¹⁹².

The organs-on-a-chip represent a promising *in vitro* platform for tissue modelling due to their accurate control of fluid flows to cultured cells in a well-defined and reproducible manner¹⁹³. Organs-on-a-chips provide spatio-temporal gradients of nutrients, mechanical strain and vasculature mimicking the interstitial flow. They are able also to resemble the biochemical and biomechanical cues of ECM¹⁵¹. In this context, the 3D bioprinting technique allows the production of organs-on-a-chips by organizing cells in a spatially-precise manner assuring a controlled and disease-specific tissue irroration by microfluidic devices¹⁹⁴. Thrombosis-on-a-chip¹⁹⁵, cardiac tissue-on-a-chip¹⁹⁶, lung-on-a-chip¹⁹⁷, eye-on-a-chip¹⁹⁸, renal-proximal-tubule-on-a-chip¹⁹⁹ and bone marrow-on-a-chip²⁰⁰ have been reported, as well as human tumoral bone-on-a-chip²⁰¹, a leukemia-on-a-chip²⁰² and a glioblastoma-on-a-chip model²⁰³.

The microfluidics can be also used as microreactors to dynamically seed the cells in scaffolds, overcoming the constraints of a static cell culture and stimulating the development of tissue-like constructs²⁰⁴.

1.4.Chorioallantoic Membrane (CAM) of chick embryo

The standard *in vivo* preclinical model for cancer research is the mouse²⁰⁵, which has a lot of advantages such as the wide-spread availability of xenograft mouse models²⁰⁶. However, these models require a long time to engraft the tumour in mice and complications derived by variable take-rates could be presented, increasing costs. The animal facility requires substantial investments for correct floor space, air handling, humidity and temperature control, and associated husbandry. Mostly, experiments must adhere to national animal licensing rules and they are related to ethical issues²⁰⁷.

On the other hand, avian models may offer a viable alternative to mouse xenografts. The fertilized chicken egg contains a highly vascularized membrane, named the Chorioallantoic Membrane (CAM), that surrounds the embryo and is responsible for gas exchange and nutrients by drawing calcium from the porous shell²⁰⁸. The CAM derived from the fusion between the chick allantois and the chorion on Embryonic Day of Development 6 (EDD6), namely six days post-fertilization, while the whole development of the chick embryo lasts for 21 days after which it hatches²⁰⁹.

One of the main advantages of using the CAM model for tumour grafting is the fact that the immune system of the chick embryo is not fully developed until EDD18, allowing the human tumour to establish itself in this environment in just 3-7 days; indeed, the innate immune system starts to develop from EDD3 to EDD16 while the adaptive immunity develops from EDD10 to EDD18²¹⁰. Consequently, the experiments are always synchronized with the EDDs of the chick embryo up to EDD18. The immature immune system allows also the preservation of immunogenic properties from human cells, indicating that the functionality and biological behaviour of the tumour graft could be preserved²¹¹.

Moreover, the CAM model is highly vascularized, making it suitable for studying tumour growth²¹², metastasis²¹³ and angiogenesis²¹⁴ with a high rate of successful tumour transplantation as it is naturally in an immunodeficient state until EDD18. Once the tumour is grafted, the time course and onset of vascularization inside the CAM involving an initial avascular phase of 72 hours during which the tumour growth is highly restricted²¹⁵ and the avascular tumour graft can undergo necrosis and autolysis²¹⁶. The neovascularization of the tumour begins 72-96 hours after implantation, followed by a rapid growing phase, due to the release of angiogenic factors by tumour cells, and rescue of the necrosis of the tumour²¹⁷.

Last but not least, the CAM adheres to the principles of the 3Rs (Replacement, Reduction and Refinement) as it is not recognized as a protected species until EDD17 under European law²¹⁸, making it easily suitable, economical and free of ethical issues. It has been determined that the chick embryo cannot experience pain up to EDD17, making it easy to be used for experimentation without the requirement needed for an animal protocol²¹⁹. Again, the cost of the embryonic eggs is low, the facility access is reduced and the manipulation of the eggs is easier than mice, making the CAM model an excellent alternative to standard animal models²²⁰.

2. THE AIM OF THE STUDY

The OS is the commonest primary solid malignancy of bone sarcomas, with approximately 80% of patients reporting localized primary tumour, while the other 20% present initial pulmonary metastases⁷. The overall survival rate is less than 20%⁷ and 28%²²¹ for metastatic and recurrent OS patients, respectively.

The challenges in treating the OS are due to its high heterogeneity in terms of histological subtypes²⁷, clones of tumour cells and the dynamic TME²⁸. Among tumour cells, a small percentage of CSCs living in a defined niche represents a critical subset of self-renewing tumoral clones that play a key role in achieving tumour heterogeneity²⁸. Moreover, different cell types (i.e. immune cells, MSCs, CAFs, epithelial cells), molecular components (e.g. growth factors and cytokines), blood vessels, environmental conditions (i.e. hypoxia, acidosis) and the ECM closely interact with tumour cells creating a complex TME³. MSCs have a significant role in OS origin and progression as normal MSCs⁴⁶ and, tumoral MSCs⁴⁶ and CAFs⁶², are directly “educated” into pro-tumoral phenotype by tumour cells and the rest of TME.

The current therapeutic regimen⁹⁵ does not completely eradicate the tumour mass giving rise to recurrences and metastasis. The failure of the conventional regimen is mainly due to (1) the drug resistance phenomenon, (2) the poor *in vitro-in vivo* and preclinical-clinical transition ability of drug candidates entering the DDDP and (3) the heterogeneity of the OS. These limitations may be partly related to the common use of the standard *in vitro* 2D models that show well-known limitations in resembling human tissue complexity^{135, 222}. To overcome these limitations, it is essential to develop more biomimetic and predictive 3D platforms that better resemble human tumours in a laboratory setting. These platforms can help elucidate disease mechanisms, identify potential new targets, enable reliable drug screening, and potentially reduce the use of animal models.

The scientific community is actively working to engineer *in vitro* 3D tumour models following the Tumour Engineering approach, exploiting and combining different Tissue Engineering technologies (e.g. spheroids, organoids, scaffolds, hydrogels, microfluidics)^{2, 134}. Most of the currently available models are still unripe and new advances need to be reached to strictly resemble a such complex disease.

The aim of this thesis is the design, development and biological investigation of two main groups of predictive *in vitro* 3D Osteosarcoma models (3D OS models) resembling two important active players of the OS complexity: the heterogenic cell population and the dynamic extracellular matrix. A hybrid composite hydroxyapatite-based scaffold (MgHA/Coll), derived from a biomineralization process involving the direct nucleation of magnesium-doped hydroxyapatite nanocrystals (MgHA) on self-assembling type I collagen fibrils (Coll), has been used as bone-like ECM for all the proposed

models²²³ due to its ability in resembling the bone formation process, the biochemical composition and the nanostructure of the bone tissue^{223, 224}. To recapitulate the cell population heterogeneity two different approaches were defined and implemented in the bone-mimetic scaffold.

i) The first group of 3D OS models concerns the *in vitro* reproduction of the CSCs niche by using enriched-CSCs as sarcospheres (3D CSCs niche model). The CSCs were first identified in OS growing as spherical colonies, called sarcospheres³¹. A well-established “sarcospheres-forming culture” has been thus used to enrich CSCs in the form of spheroids thanks to serum-free, non-adherent and nutritionally deficient conditions²²⁵. The 3D CSCs niche model has been optimized in two main steps: (1) the sarcospheres derived from MG63 and SAOS-2 osteosarcoma cells have been serially passaged *in vitro* in order to select the most promising sarcospheres generation (Gs) in terms of stem and tumoral properties. Indeed, the serial passage of spheroids has been recently shown to increase their undifferentiated features, sphere-forming efficiency and tumoral properties in various tumours^{226, 227}; (2) two different injection-based approaches, called open and close injection, have been designed and developed to seed the sarcospheres into MgHA/Coll scaffold to resemble the TME of CSCs niche at 360 °C, addressing the well-known limitations of standard cell seeding on scaffolds surface. Through the selection of the optimal Gs and the use of two precise cell seeding procedures, advanced *in vitro* 3D CSCs niche models were obtained after 7 days of culture, showing promising tumorigenic properties *in vivo* CAM models.

Then, the validated 3D CSCs niche model was finally used to evaluate the drug resistance phenomenon by performing *in vitro* and *in vivo* CAM model exploration treating the models with different common chemotherapeutics (e.g., Cisplatin and Doxorubicin) and a promising organic Se compound (e.g. Dibenzyl diselenide – DBDS¹¹³) for 96 hours treatment. A deeper biological evaluation of the 3D CSCs niche model after chemotherapeutic treatments was performed with the final aim of investigating the ability of our model to recapitulate the drug resistance phenomenon occurring *in vivo*.

ii) The second group of *in vitro* 3D OS models has been focused on the study of the crosstalk between osteosarcoma cells and MSCs. It is well-established that the MSCs contributes to the origin and progression of OS through their “education” in tumoral MSCs and CAFs following the interaction with TME and, tumour cells⁴⁶. In turn, MSCs-released cytokines are suggested to improve the stem-like phenotype and aggressiveness of tumour cells^{58, 59, 61}, favouring tumour progression. Many questions remain about the real impact of MSCs on OS and studies exploiting the use of a 3D bone-like ECM are still unexplored. Facing these limitations, a 3D Co-culture model was designed and developed by implementing GFP-MNNG HOS OS cells and BM-MSCs (iBM-MSCs) seeded at 1:1 ratio on MgHA/Coll scaffold. The 3D Co-culture model was investigated *in vitro* after 7 and 14 days

of culture; the cellular ability in colonizing the scaffold, together with the cell morphology, MMPs and related TIMPs, and gene and protein expression of tumoral and CAFs-related markers were evaluated in order to investigate the potential ability by tumour cells in inducing MSCs malignant transformation and, in turn, the increase aggressiveness of tumour cells in the presence of MSCs, compared to mono-culture and scaffold-free conditions.

All these 3D OS models allowed the investigation of the cellular behaviour in relation with a bone-like ECM and other cell types, offering promising prospects for the future development of more sophisticated systems able to develop a human-like tumour *in vitro* and *in vivo*.

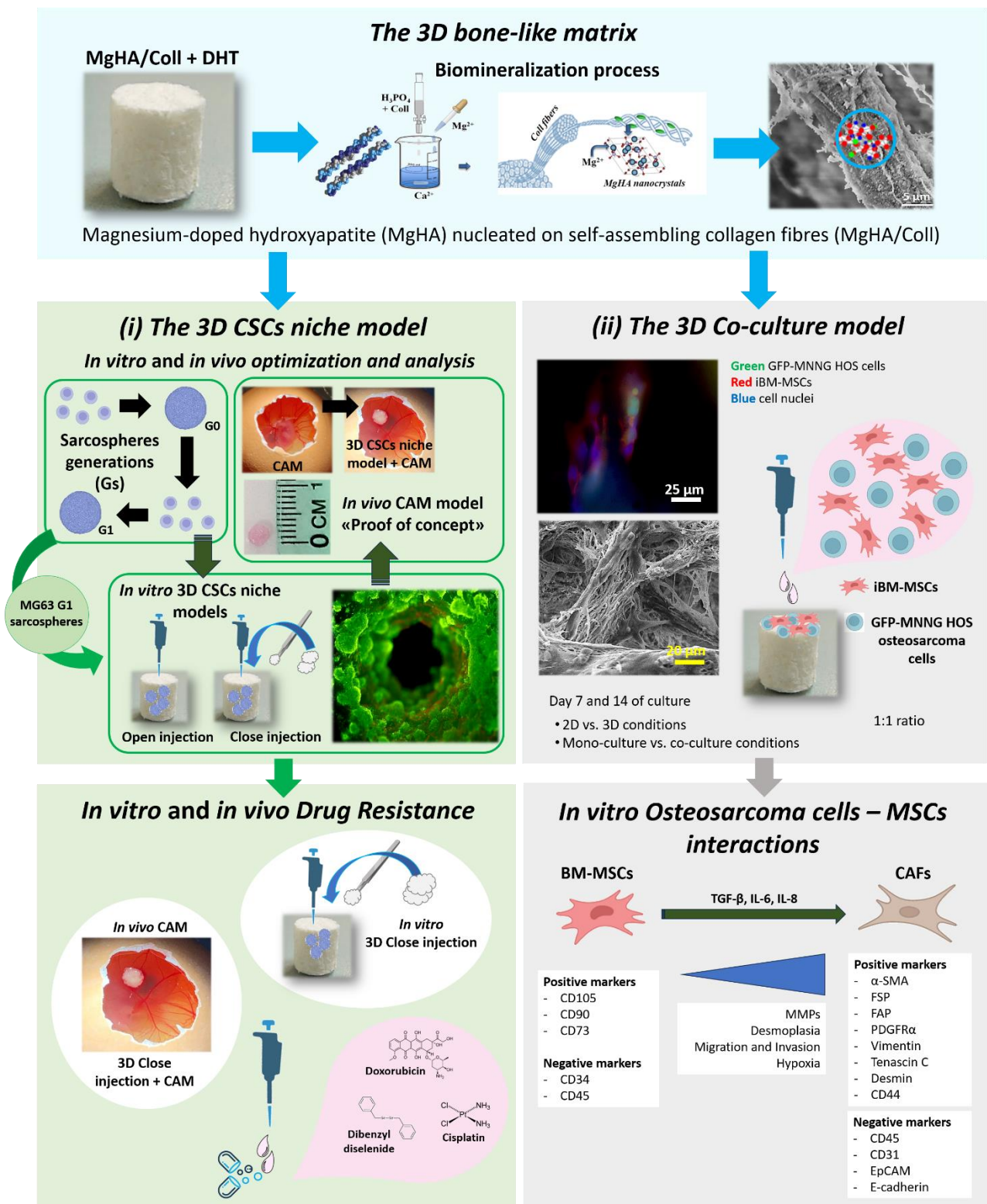


Figure 2. Representative panel of the aim of the study. (i) The 3D CSCs niche model and (ii) the 3D Co-culture model developed in this study are graphically reported, together with a brief description of the MgHA/Coll scaffold used as 3D bone-like matrix.

3. EXPERIMENTAL SECTION

3.1. Cell culture

Human Osteosarcoma MG63 and SAOS-2 cell lines were purchased from American Type Culture Collection (ATCC CRL-1427 and ATCC HTB-85, respectively) and used to develop the *in vitro* 3D CSC niche model of OS. MG63 cells were cultured in DMEM/F-12 GlutaMAX (Gibco), supplemented with 10% Foetal Bovine Serum (FBS) (Gibco) and 1% Penicillin/Streptomycin mixture (Pen/Strep) (Gibco). SAOS-2 cell line was cultured in McCoy's 5 Modified Medium (Gibco), supplemented with 15% FBS and 1% Pen/Strep.

For the development of the 3D Co-culture model of OS, Human MNNG HOS osteosarcoma cell line transfected with Green Fluorescent Protein (GFP) (GFP-MNNG HOS) were co-cultured with Immortalized Human Bone Marrow-derived Mesenchymal Stem cells (iBM-MSCs). Both cell types were kindly provided by Prof. Dominique Heymann of the Tumour Heterogeneity and Personalized Medicine Lab of the Institut de Cancérologie de l'Ouest (ICO), in Nantes, France, and cultured in DMEM high glucose (Lonza) supplemented with 5% FBS, 1% L-glutamine and 1% Pen/Strep.

Cells were grown in incubator at 37°C, 5% CO₂ and controlled humidity conditions. Once the confluence has been reached, the cells were detached from the flask by trypsinization and centrifuged. The supernatant was removed and the pellet was resuspended in fresh media. The cell number and viability were assessed by Trypan Blue Dye Exclusion Test and all cell handling procedures were performed in sterile conditions under laminar flow hood.

3.2. The Sarcosphere-forming culture

For the development of the 3D CSCs niche model of OS, the enriched-CSCs as sarcospheres were obtained from MG63 and SAOS-2 undergoing the Sarcosphere-Forming Culture³¹. Briefly, the cells were cultured in anchorage-independent conditions using Ultra-Low Attachment (U.L.A.) multi-well plates (Corning) in serum free DMEM/F12 GlutaMAX medium supplemented with 1% Pen/Strep and a specific mix of factors composed of 10 µL/mL N2 (Gibco), 20 µL/mL B27 (Gibco), 0.1 µL/mL hFGFb (Invitrogen) and 0.01 µL/mL Recombinant Human Epidermal Growth Factor (hEGF) (PeproTech). The cell culture was supplied with the mix every 2/3 days. The combination of a non-adhesive substrate, the absence of serum which typically has a differentiation effect¹⁴³ together with the addition of specific growth factors, guarantee the partial enrichment of CSCs and the support of CSCs proliferation, spheroid diameter increasing and stemness¹⁴⁴. The cell cultures were incubated at 37°C, 5% CO₂ and controlled humidity conditions for a total of 10 days to obtain the sarcospheres.

3.3. MgHA/Coll scaffold as bone-like extracellular matrix

3.3.1. The synthesis of the MgHA/Coll scaffold

The MgHA/Coll scaffold has been designed and synthesized by the Institute of Science, Technology and Sustainability for Ceramics (ISSMC) of the National Research Council²²³ (CNR), in Faenza, Italy, and it was obtained by the reproduction in a laboratory setting of *in vivo* biomineralization process which occurs during bone formation. The process is biologically-inspired and highly biomimetic and, mostly, the biomineralization step is driven by the progressive decrease of pH where the pH neutralization induces two different processes at the same time: (1) the nucleation of the inorganic phase and (2) the self-assembling of the organic matrix. The Magnesium-doped hydroxyapatite (MgHA) represents the inorganic phase, as a mineral component of the bone, that is directly nucleated onto the self-assembling type I collagen fibres (Coll), as the main organic component of the bone tissue, creating a 3D highly interconnected structure.

An acid aqueous suspension composed of 150 g of type I collagen gel (Typeone Biomaterials S.r.l., Calimera, LE, Italy) dissolved into phosphoric acid solution (2.41 g in 500 mL; H₃PO₄, 85 wt. %, Sigma Aldrich) was prepared at room temperature. A basic aqueous suspension composed of 0.35 g of magnesium chloride (MgCl₂·6H₂O, 99 wt. %, Sigma Aldrich) and a calcium hydroxide suspension (2.71 g in 500 mL; Ca(OH)₂, 95 wt. %, Sigma Aldrich) was prepared at room temperature. The, the acid suspension was dropped into the basic one under stirring conditions at 25 °C, during which the pH neutralization induces the nucleation of MgHA nanocrystals onto self-assembling collagen fibres, forming the MgHA/Coll hydrogel. The hydrogel was matured for 2 hours at 25 °C, washed and the 48 hours freeze-drying (– 40 °C and + 25 °C) under 0.086 mbar vacuum conditions (5 Pa, LIO 3000 PLT) were performed to obtain a material with defined pores morphology, dimension distribution and interconnected porosity. The scaffolds were stabilized, preserving the biological cues of the collagen and avoiding poor mechanical resistance, by dehydrothermal (DHT) crosslinking treatment at 160 °C for 48 hours under a pressure of 0.01 mbar. The scaffolds (ø: 9mm; h:6mm) were sterilized by 25 kGy γ-ray irradiation and used in these biological studies²²⁸.

3.3.2. The morphological analysis

The morphology of the scaffold is fundamental to guarantee a good cellular adhesion, viability and colonization. Specifically, the scaffold must satisfy some important parameters including the average size of the pores, their distribution, their volume, the interconnection, the shape and the roughness of the pore wall²²⁹, to facilitate cellular behaviours.

The morphology was analysed by Scanning Electron Microscopy (SEM) to investigate the inner structure and porosity degree of the material. The SEM provides significantly enlarged images of the sample's surface (up to 200000X) with high resolution and depth of field, superior to those that can

be obtained from an optical microscope. The image derived from the scan of the sample's surface by a thin primary electron beam generated from a tungsten cathode; part of the primary electrons is reflected without decreasing the initial energy giving rise to the so called backscattered electrons (BSE), while secondary electrons (SE) represent electrons which escape from the sample due to their diffusion ability derived from the energy received from primary electrons. Therefore, not reflected primary electrons and secondary electrons only escape with low energy (energy < 50 eV). In addition, x-ray photons are emitted following the expulsion of the inner shell electron from the sample by the primary beam, giving information on the elemental composition of the sample²²⁹.

In this context, SE are detected by a scintillator photomultiplier device and processed into a two-dimensional intensity distribution generating the digital image of the sample based on variations in the emission intensity, namely providing information on the surface morphology and the angle of incidence formed with the beam. The number of SE in the detector and the electron spot size determine the brightness and the spatial resolution of the image, respectively. In addition, the BSE may be used alone to form an image or in combination with x-rays which is significantly affected by the atomic number of the element involved, thus providing information mainly on the composition of the sample which contributes to the contrast of the image²²⁹.

For the analysis to be effective, the sample must be made conductive through the sputtering technique, which allows the surface of the sample to be coated with a thin metal film²²⁹. Therefore, for the study²²⁸, the scaffolds were mounted onto aluminium stubs using black carbon tapes and coated with gold, then they were analysed using high resolution environmental scanning electron microscopy (ESEM) (ESEM Quanta 200, FEI, Thermo Fisher Scientific Inc.) under a pressure of 0,1 mTorr at an accelerating voltage of 7 or 10 kV.

The size of the pores is also relevant because if the pores are too small they risk hindering cell penetration, the production of extracellular matrix and the vascularization of the internal regions of the scaffold²²⁹. The scaffolds total porosity was calculated by density method²³⁰ according to the formula:

$$Total\ porosity\ (\%) = 100 - \left(\frac{\rho}{\rho_{theoretical}} \times 100 \right)$$

where ρ is scaffold density determined with the following equation:

$$\rho = W / \left(\pi \times \left[\left(\frac{D}{2} \right)^2 \right] \times H \right)$$

in which W is weight, D is diameter and H is height of the scaffold.

The theoretical density of the material is calculated from the theoretical density and weight fraction (XA, XB etc.) of each reagent in the following way:

$$\rho_{theoretical} = (\rho_{theoretical}(A) \times XA) + (\rho_{theoretical}(B) \times XB)$$

All values were expressed as the mean $\pm$ SEM (N=3).

The macroporosity percentage was calculated by the water squeezing method²³¹. Briefly, the scaffold was equilibrated in deionized water for one hour and weighed ($M_{swollen}$), then squeezed to remove the water filling pores and weighed again ($M_{squeezed}$). Macropores volume was calculated using the following equation:

$$\text{Macroporosity (\%)} = \frac{M_{swollen} - M_{squeezed}}{M_{swollen} \times \rho} 100$$

All values were expressed as the mean $\pm$ SEM (N=3).

3.3.3. Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES)

The ICP-OES is a method of optical emission spectrometry that exploits the emission spectra of the sample's molecules to identify and quantify the multiple elements present with high sensitivity, like parts per billion (ppb) to parts per million (ppm), with very little interference²³². The technique is based upon the spontaneous photons emission from ions or atoms whose excitation takes place in a radio frequency discharge²³².

The instrument has two parts, the inductively couple plasma and the optical emission spectrometer²³³. The samples are introduced as liquid form into inductively coupled argon plasma through an injection system, here molecules are atomized and ionized in the plasma and excited²³³; specifically, while the role of the argon is to transform the liquid sample in the form of aerosol, though the so called aerosol nebulization process, followed by aerosol vaporization and drying by high temperatures in order to release the elements as free atoms, the plasma then ionizes and excites the atoms to convert them into ions and promote their excited state. The return to a low energy level from an excited energy level by electrons causes the release of photons by ions and atoms, called ionic or atomic emission, that are then collected by a detector able to separate different emission wavelengths from each other. The detector measures the emission signal intensities of the respective wavelengths, that corresponds to the energy difference between the excited state and ground state of the electron; the emission signal strength is proportional to the element's abundance in the plasma. Standard solutions allow to create a calibration curve to evaluate the element concentration²³³.

The quantitative determination of magnesium (Mg^{2+}), calcium (Ca^{2+}) and phosphate (PO_4^{3-}) ions that constitutes the inorganic mineral component of the MgHA/Coll scaffold was assessed in this study²²⁸ by ICP-OES (Agilent Technologies 5100 ICP-OES, Santa Clara, USA). Briefly, 40 mg of sample was dissolved with 2 ml nitric acid (65 wt.%), sonicate and diluted in 100 ml of milli-Q water.

3.3.4. Thermogravimetric analysis (TGA)

The TGA allows to monitor the mass (weight) of the material as a function of the temperature or time as the sample undergoes to a controlled temperature program in a controlled atmosphere, namely the

furnace. The furnace consists of a sample pan supported by a precision balance and the pan is heated or cooled during the experiment, during which the weight of the sample is monitored. A sample purge gas controls the sample environment; the gas may be inert or a reactive gas that flows over the sample and exits through an exhaust to remove gaseous by-products derived from material's reactions²³⁴.

In this study²²⁸, the MgHA/Coll scaffolds were analysed by TGA using STA 449/C Jupiter (Netzsch, Germany) on 10 mg of sample placed in alumina crucible under airflow, brought from room temperature to 1100°C at a heating rate of 10°C/min. The data are plotted as a thermal curve with the time or temperature on the abscissa (X-axis) and the weight (mg) or the weight percentage (%) on the ordinate (Y-axis).

3.3.5. X-Ray Diffraction (XRD)

The XRD analysis is a non-destructive technique that provides detailed information about the crystallographic structure, chemical composition, chemical bonds, atomic position and physical properties of a crystalline material²³⁵. The technique is based on the constructive interference between monochromatic X-rays and a crystalline sample. X-rays are short wavelength electromagnetic radiation generated by the deceleration of electrically charged particles with sufficient energy. In XRD, X-rays are collimated and directed to the crystalline sample, where the interaction of the X-rays with the sample's structure produces a mean of incident X-rays to diffract in specific directions, namely diffraction rays, which are detected, processed and counted. The measure of the angles and intensities of the diffracted rays are detected and plotted to display a unique diffraction pattern, which is a sum of diffraction patterns of each phase; indeed, each phase of the crystalline material produces a unique diffraction pattern due to its specific chemistry and atomic arrangement. Indeed, imperfection in the sample, such as the composition heterogeneity, crystal structure defects, microstains and crystallize size, would affect the pattern of the diffracted signal²³⁵.

In this study²²⁸, the XRD patterns of MgHA/Coll scaffolds were recorded by a Bruker (Karlsruhe, Germany) AXS D8 Advance diffractometer, in reflection mode with CuK α radiation ($\lambda = 1,54178 \text{ \AA}$) generated at 40 kV and 40 mA and equipped with a Lynx-eye position-sensitive detector. XRD spectra were recorded in the 2θ range from 20° to 60° with a step size (2θ) of 0,02° and a counting time of 0,5 seconds.

3.3.6. Fourier Transform Infrared spectroscopy in Attenuated Total Reflection mode (FTIR-ATR)

FTIR spectroscopy is a semi-quantitative technique that exploits the infrared radiation to determine what fraction of the incident light is absorbed at a specific wavelength, providing a spectrum of the vibration of the bonds within a molecule, for analysing the chemical structure of various materials.

Each spectrum represents the chemical fingerprint for the identification of the chemical structure of the sample²³⁶.

The FTIR-ATR specifically is a powerful analytic technique used to investigate the molecular composition of a material by combining the principles of infrared spectroscopy, which provide the chemical structure of the material, with the ATR mode to gain information about the chemical bonds and the functional groups present in the sample. This technique measures the absorption of infrared light by the sample placed in contact with a high-refractive-index crystal, through a crystal-sample interface interaction; a portion of the infrared light is absorbed by the sample, while remaining light is reflected. Shifts in the frequency of absorption bands and changes in relative band intensities indicate changes in the chemical structure or changes in the environment around the sample. Therefore, the FTIR-ATR spectrometry can be used to determine the surface chemistry following induced chemical or physical modifications²³⁶.

A flake of freeze-dried MgHA/Coll scaffold was used in this study²²⁸ to perform the FTIR-ATR using a Ni-colet iS5 spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) with a resolution of 2 cm⁻¹ by accumulation of 64 scans covering the 4000 to 400 cm⁻¹ range, using a diamond ATR accessory model iD7.

3.3.7. Swelling and degradation behaviour

The swelling test is used to investigate the ability of a material to absorb liquids. The term “swelling” means the increase in the volume and weight of a solid caused by the absorption of liquids or vapours present in the environment. The swelling phenomenon is caused by a diffusion process which can be accompanied by a solvation process, giving also a qualitative measure of the degree of hydrophilicity of the material: the higher the swelling, the more hydrophilic the material will be and vice versa²²⁹.

There are two types of swelling, limited and unlimited; in the first case, the macromolecules of the material are strongly bound and the material reaches a certain absorption limit, stopping the swelling and preserving the solid-liquid interface constant. The unlimited swelling occurs in the materials characterized by weak bonds, in which absorption is continuous and the interface between solid and liquid changes continuously.

In this study²²⁸, the Swelling Ratio (Qs) of the MgHA/Coll scaffold has been defined to evaluate the fractional increase in the weight of the material due to water absorption²³⁷. Briefly, the scaffold was immersed in PBS 1X at pH 7.4 with 0,1% (w/v) of NaN₃ at 37°C, the excess of water was removed at predetermined time points with a piece of absorbent paper and the sample was weighted. The swelling ratio (Qs) was evaluated through equation:

$$Qs = \frac{Ws - Wd}{Wd}$$

where W_s was the weight of the swollen sample and W_d was the initial weight of the dried sample ($N=3$).

The Degradation Percentage (D) of the scaffold was also calculated by immersion-based method²²⁸. Briefly, the scaffolds were immersed in the PBS 1X at 37°C and, at predetermined time points, they were washed twice with milliQ water, freeze-dried and weighed. The D was evaluated using the following equation:

$$D (\%) = \frac{W_i - W_f}{W_i} \times 100$$

where W_i was the weight of the dried sample and W_f was the weight of the freeze-dried sample after degradation at a specific time point ($N=3$).

3.4.The development of the 3D CSCs niche model

3.4.1. Serial sarcospheres generations

3.4.1.1.The development of the sarcospheres generation

In this study²²⁸, serial sarcospheres generations (Gs) were performed and deeply characterized *in vitro* as follows. After the 10 days of Sarcosphere-Forming Culture, the obtained sarcospheres, called here as generation 0 (G0) were passaged *in vitro* to obtain the next generation, namely the generation 1 (G1). Different works report the improvement of stemness properties by spheroids' serial passages^{226, 227}. Briefly, the G0 was collected, centrifuged and a combination of enzymatic and mechanical dissociation was performed to obtain single cells, which were counted and seeded again under Sarcosphere-Forming Culture for 10 days to form G1. Sarcospheres were serially passaged for a total of 4 Gs here reported as G0, G1, G2 and G3 for MG63 and SAOS-2 cells. The Gs were investigated *in vitro* in terms of proliferation, sphere-forming efficiency (SFE), size distribution, gene expression, Stem Cell Frequency (SCF) and, migration and invasion ability, and the most promising Gs has been selected to be used for the optimization of the 3D scaffold-based seeding.

3.4.1.2.The PrestoBlue Cell Viability Reagent

The Presto Blue Cell Viability reagent (Invitrogen) was used to assess cell viability and proliferation of Gs of MG63 and SAOS-2²²⁸. Single cells of each generation were cultured in U.L.A 96 well-plates under Sarcosphere-Forming Culture conditions at a density of 15,000 cells/cm². At 1, 3, 7, and 10 days of culture, the assay was performed following the manufacturer's instruction. Briefly, the sarcospheres were incubated with 10% (v/v) Presto Blue reagent for 2 hours at 37 °C, 5% humidity and CO2 conditions and the content/well was collected, centrifuged and read by Fluoroskan Microplate Fluorometer (Thermo Fisher Scientific) at 544 nm excitation and 590 nm emission. For each time point, six biological replicates of each Gs were analyzed ($N = 6$).

3.4.1.3. Sarcospheres-Forming Efficiency (SFE) and size distribution

SFE and size distribution of each Gs of MG63 and SAOS-2 cells were investigated²²⁸. Briefly, single cells of each generation were cultured in U.L.A. 96 well-plates under Sarcospheres-Forming Culture conditions at three different densities: 1,000 cells/well, 3,000 cells/well and 6,000 cells/well. The SFE and size distribution were evaluated after 10 days of culture by the randomic acquisition of 10 fields/well at 10X magnification at the optical microscope (Nikon).

The SFE was calculated as percentage (%) of the number of sarcospheres in each well divided by the cell density:

$$\left[\left(\frac{N^{\circ} \text{ sarcospheres}}{\text{cell density}} \right) \times 100 \right]$$

The size distribution was also assessed as a percentage (%) with respect to the total number of sarcospheres according to a dimensional range (50-100 µm, 101-200 µm, > 200 µm diameter). The diameter of the sarcospheres was measured and analysed by using the ImageJ software (ImageJ 1.53t), considering only sarcospheres with ≥ 50 µm diameter.

One experiment was performed with a biological triplicate for each generation at each cell density (N=3).

3.4.1.4. Stem Cell frequency (SCF)

Limiting Dilution Assay (LDA) was performed in order to assess the frequency of one stem cell per each MG63 Gs²²⁸. For each Gs, single cells were serially diluted at a final density of 1000, 500, 250, 50, 10 and 1 cell/well in U.L.A. 96 well-plates and incubated 14 days under Sarcospheres-Forming Culture conditions. For the analysis, the count of the number of wells per density containing sarcospheres was performed at the optical microscope only considering sarcospheres with ≥ 50 µm diameter. The analysis was performed by exploiting the Extreme Limiting Dilution Analysis (ELDA) software (<https://bioinf.wehi.edu.au/software/elda/>)²³⁸ entering the dose (cell density), the tested (N° of cultures tested), the response (N° of positive culture containing sarcospheres) and the group (the group to which the sarcospheres belong, namely the generation number) data. The results were plotted in a graph showing the corresponding trend line of linear regression; the Y axes showed the log fraction non-responding, the number of seeded cells/well is reported on X axes, while the log-active cell fraction in each Gs was represented by the slope of the trend line. The 95% confidence interval was represented by the dotted lines and the down-pointing triangle is the data value with zero negative response. Two experiments were performed for a total of 16 biological replicates for each cell density for each generation (N=16).

3.4.1.5. Migration and invasion analysis

The Transwell Inserts for 24 well-plates with 8.0 μm pore size membrane (Corning) were used to investigate the migration and invasion ability of MG63 Gs²²⁸. For the migration analysis, single cells of each Gs were seeded at a density of 100,000 cells/ cm^2 in the upper chamber of the insert under serum-free conditions, while the chemoattractant was represented by the 20% FBS in the lower chamber. For the invasion analysis, 100 $\mu\text{L}/\text{cm}^2$ Matrigel GRF (Growth Factor Reduced Basement Membrane Matrix, Corning) coating in serum-free media has been performed on the insert membrane by 30 minutes' incubation at 37 °C before cell seeding. At 96 hours culturing, a wet cotton swab was used to remove the cells that failed to migrate or invade the pores of the membrane, thus remaining on the upper chamber of the insert, while 4% paraformaldehyde (PFA) was used to fix the cells that colonized the lower surface of the membrane. The fixed cells were permeabilized in 0.1% Triton-X 100 and stained with 0.5% Crystal Violet solution in order to assess the number of migrating/invasive cells; the random acquisition of 6 fields/membrane at 10X magnification at the optical microscope was performed and ImageJ software was used to count the number of cells.

For each generation, two biological replicates were performed for both migration and invasion (N=2).

3.4.1.6. Gene expression

The gene expression was analysed by Real Time PCR, following the manufacturer's instructions²²⁸. For the Gs of MG63 and SAOS-2 cells, single cells of each generation were cultured in U.L.A. 6 well-plates with a density of 5,000 cells/ cm^2 and incubated under Sarcospheres-Forming Culture for 10 days. The sarcospheres were collected, centrifuged and homogenized in Tri Reagent (Invitrogen) combined with mechanical dissociation. The RNA extraction and purification were performed by Direct-zol RNA MiniPrep Kit (Zymo Research), following the manufacturer's instructions. At this step, the samples were combined two by two into a single Zymo-Spin IICR Column to increase the RNA yields. The RNA quantification and purity degree were analysed at the NanoDrop One Microvolume UN-Vis Spectrophotometer (Thermo Fisher Scientific), following the manufacturer's instructions. The RNA was reverse transcribed into a single strand of cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem) starting from 500 ng of purified RNA, following the manufacturer's instructions. The Real Time PCR was performed using TaqMan Gene Expression Assay Kits (Applied Biosystem). Octamer-binding transcription factor 4 (OCT-4, Hs00999632_g1), Homeobox protein NANOG (NANOG, Hs04399610_g1) and Sex determining region Y-box (SOX-2, Hs01053049_s1) were analyzed to evaluate the stem properties of Gs, by using G0 as control group. Actin- β (ACTB, Hs01060665_g1) was used as housekeeping gene. One experiment was performed and three biological replicates were obtained for each condition (N = 3). Each biological replicate was analysed in three technical replicates (N=3). Data were collected from

QuantStudio 1 Real Time PCR System (Applied Biosystem) and the relative quantification of target genes was assessed by Comparative Threshold (CT) method ($\Delta\Delta CT$) where relative gene expression level equals to $2^{-\Delta\Delta CT}$ ²³⁹.

3.4.1.7. Actin and DAPI staining

The fluorescent Actin and DAPI staining was performed to evaluate the morphology of MG63 G1, following the manufacturer's instructions²²⁸. Briefly, the sarcospheres were fixed in 4% PFA and were embedded in OCT (Optimal Cutting Temperature Compound, Histo-line laboratories) after undergoing a cryopreservation process in solutions of increasing sucrose concentration (i.e. 10, 20 and 30% sucrose) in PBS 1X for at least 18 hours' incubation each. Then, the samples were cryosectioned in 5 μm sections by cryostat (Histo-line Laboratories). The sections were permeabilized in 0.1% Triton-X 100 in PBS 1X and Rhodamine Phalloidin Actin red 555 ready probs (Invitrogen) staining was used for F-actin filaments detection. DAPI staining was used as cell nuclei counterstaining. Fluor Save Reagent was used to mount the sections and images were acquired at the Inverted Ti-E Fluorescence Microscope using TRITC and DAPI filters. One biological replicate was performed (N=1).

3.4.1.8. Statistical analysis

Statistical analysis was performed by using GraphPad Prism Software (8.0.1 version). The PrestoBlue Cell Viability Reagent results are plotted in the graph as mean normalization respect to day 1 $\pm$ SEM and they were analysed by Two-Way analysis of variance (Two-way ANOVA) and Tukey's multiple comparisons test²²⁸.

The SFE were plotted in the graph as mean SFE values $\pm$ SEM, while size distribution was reported as percentage (%) distribution of sarcospheres diameter/total sarcospheres $\pm$ SEM; the statistical analysis was performed by Two-Way ANOVA and Tukey's and Sidak's multiple comparisons tests²²⁸.

The Stem Cell Frequency data was analysed by ELDA software (<https://bioinf.wehi.edu.au/software/elda/>) assuming the Poisson Distribution. The Pairwise test for differences between coupled generations and by Chi-Square test as an overall test for differences between any of the generations were performed²²⁸.

The results of migration and invasion evaluation were plotted in the graphs as mean of counted cells $\pm$ SEM and they were analysed by One-Way analysis of variance (One-Way ANOVA) and Tukey's multiple comparisons test²²⁸.

The results of qRT-PCR are plotted in the graphs as fold-change expression relative to the experimental control $\pm$ SEM and they were analysed by Two-Way ANOVA and Tukey's multiple comparisons test. *p value ≤ 0.05 , ** p value ≤ 0.01 , ***p value ≤ 0.001 and ****p value ≤ 0.0001 ²²⁸.

3.4.2. The optimization of sarcospheres seeding into the 3D MgHA/Coll scaffold

3.4.2.1. The development of the 3D injection-based seeding approach

The seeding of the selected generation of sarcospheres into the MgHA/Coll scaffold has been optimized in this study with the final aim of obtaining a close TME at 360°C, facing the limitations reported by the standard cell seeding on scaffolds²²⁸. Addressing these drawbacks, here two different injection-mediated approaches of Gs seeding in MgHA/Coll scaffold ($\sim \varnothing$:9 mm; h: 6 mm) were designed and developed as graphically reported in Figure 3. Briefly, the MgHA/Coll scaffold was pre-conditioned in serum-free media for 24 hours before the sarcospheres seeding to make it to swell and absorb cell media. The MG63 cells were seeded at a density of 50.000 cells/well in U.L.A. 6 well-plates and cultured as previously reported (see paragraph 3.4.1.1.) to obtain G1 sarcospheres. For the 3D seeding, the MG63 G1 of each well were separately collected, centrifuged and seeded by vertically injecting 20 μ L drop/scaffold in the core of the material using a p200 tip. One group of 3D CSCs niche model, labelled as “close injection”, has been further optimized by manipulating wet flakes of MgHA/Coll material with the use of tweezers as a cap to close the scaffold hole derived from the injection procedure; in a second group, labelled as “open injection”, no caps were used. After the seeding, 20 minutes' incubation was performed to allow sarcospheres pre-adhesion to the scaffold before cell media addition. Both groups of 3D CSCs niche model were kept under “Sarcospheres-Forming Culture” for a total of 7 days at 37°C, 5% CO₂ and controlled humidity. The two groups were deeply analysed *in vitro* in terms of sarcospheres colonization, gene expression and protein expression of CSC- and niche-related markers, and tumorigenic properties *in vivo* CAM models.

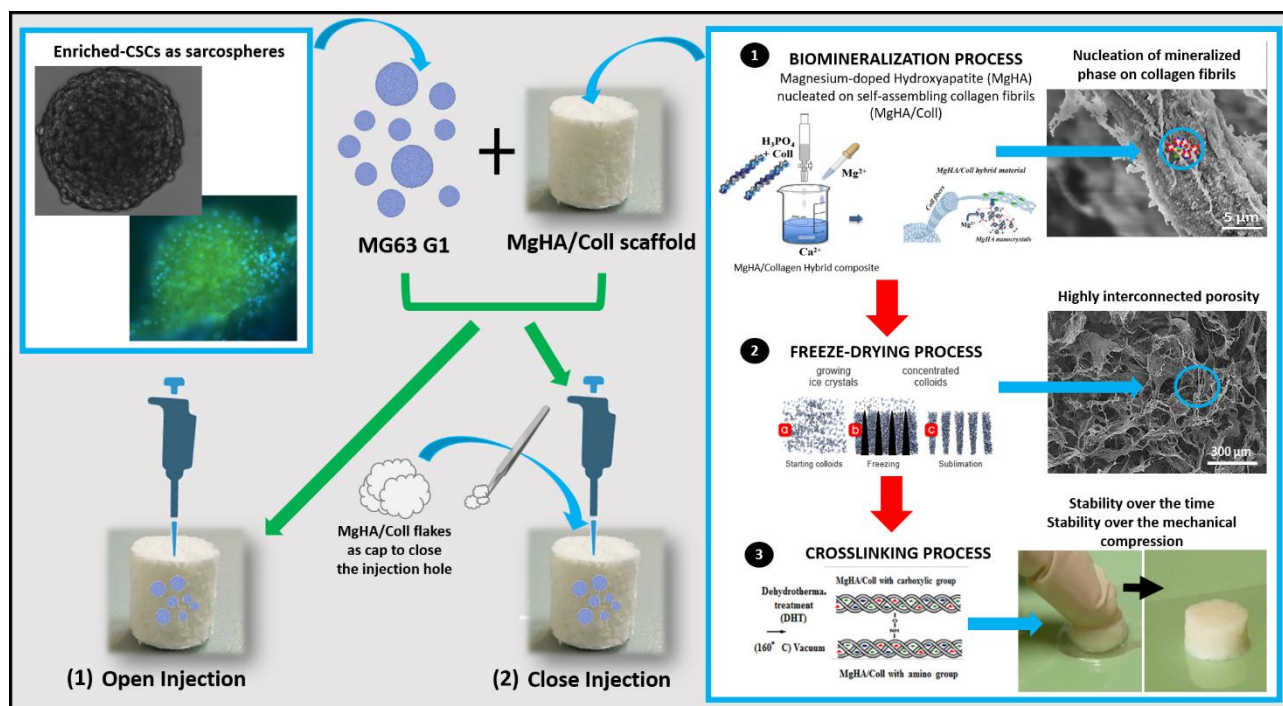


Figure 3. Graphical representation of the optimization of the MG63 G1 seeding into 3D MgHA/Coll scaffold. The development of the 3D CSCs niche model by two injection-based approaches, here reported as Open and Close Injection, is graphically reported starting from the original MgHA/Coll scaffold, whose synthesis process is resumed²²⁸.

3.4.2.2. Live and Dead Assay

The MG63 G1, selected as the most promising generation, was used for the optimization and development of the 3D CSCs niche model²²⁸. The viability of MG63 G1 after injection-based seeding into the MgHA/Coll scaffold to create the 3D CSCs niche model was preliminarily analyzed by Live and Dead Assay, following the manufacturer's instructions to assess the healthy status and morphology of sarcospheres. At 3, 7 and 14 days of culture, the 3D CSCs niche model was washed in PBS 1X and incubated for 15 minutes at 37°C in the dark with the Live and Dead solution composed of 2 μ M Acetoxymethyl Calcein (AM-calcein) and 4 μ M Ethidium Homodimer-1 (EthD-1) in PBS 1X. The images were acquired at the Inverted Ti-E Fluorescence Microscope (Nikon) using FITC and TRITC filters for green calcein and red ethidium detection for live and dead cells, respectively. For the experiment, one biological replicate was performed (N=1).

3.4.2.3. Gene expression

The gene expression was analysed by Real Time PCR, following the manufacturer's instructions (see paragraph 3.4.1.6)²²⁸. Each 3D CSCs niche model was separately collected and homogenized in two steps of 300 μ L Tri Reagent by pestle-mediated breaking and vigorous vortexing. For the RNA extraction and purification, the Direct-zol RNA MiniPrep Kit was used by combining the samples two by two into a single Zymo-Spin IICR Column to increase the RNA yields. For the RNA quantification and the RNA reverse transcription into cDNA see paragraph 3.4.1.6. The Real Time

PCR was performed using TaqMan Gene Expression Assay Kits for OCT-4, NANOG and SOX-2 to evaluate the preservation of the stemness properties by G1 after 3D seeding (see paragraph 3.4.1.6.). Hypoxia Inducible Factor 1 alpha (HIF-1 α , Hs00153153_m1), Interleukin 6 (IL-6, Hs00174131_m1) and Notch homolog 1, translocation-associated (NOTCH-1, Hs01062014_m1) were also analysed as CSC niche-related markers. The scaffold-free sarsospheres were used as control group and Actin- β was used as housekeeping gene (see paragraph 3.4.1.6.). One experiment was performed and three biological replicates were obtained for each condition (N = 3). Each biological replicate was analysed in three technical replicates (N=3). For the data collection and elaboration see paragraph 3.4.1.6²³⁹.

3.4.2.4. Histological and immunohistochemical analysis

The CSCs niche model was fixed in 4% formalin for 15 minutes at room temperature and embedded in paraffin to perform histological and immunohistochemical analysis²²⁸. Briefly, the 3D CSCs niche model was dehydrated in an increasing scale of alcohol solutions (from 70 to 100% v/v) and cleared in xylene reagent (Sigma) under vacuum conditions. Then, the sample was embedded in paraffin in metal cells and 5 μ m sections were performed using a semi-automatic rotary microtome (Histo-Line Laboratories). Before the histological or immunohistochemical analysis, the sections were deparaffinized at 59°C, cleared in Xylene, and hydrated in a decreasing scale of alcohol solutions (from 100 to 70% v/v) and water.

The immunohistochemical analysis was performed by using the UltraTek HRP (Horseradish peroxidase) Anti-Polyvalent (AEC) Staining System (Histo-line Laboratories) on the 3D CSCs niche model, following the manufacturer's instructions, and the Nuclear Fast Red (Sigma Aldrich) as counterstaining for cell nuclei identification. Briefly, the sections were incubated with primary antibodies for SOX-2 (1:200, MA1014, Thermo Fisher Scientific), OCT-4 (2 μ g/mL, MA1104, Life Technologies), NANOG (1:50, MA1017, Thermo Fisher Scientific), HIF-1 α (1:800, 36169S, Cell Signaling Technology) and NOTCH-1 (1:200, 4380S, Cell Signaling Technology), following the datasheet instructions. One biological replicate for each condition was used (N=1).

For histological analysis, the Haematoxylin (Fluka, Sigma Aldrich) and Eosin (Histo-Line Laboratories) (H&E) staining was performed on the sections, following the manufacturer's instructions.

After the application of the immunohistochemistry kit or histological staining, the sections were dehydrated in increasing scale of alcohol solutions (from 70 to 100% v/v), cleared in xylene and mounted. Images were acquired at the Optical Microscope.

3.4.2.5. Dynamic Mechanical Analysis (DMA)

The mechanical properties of the 3D CSCs niche model were evaluated in terms of viscoelastic behaviour by using the Dynamic Mechanical Analysis (DMA) using the DMA Q800 (TA Instruments). The DMA is a thermal analysis technique during which the sample undergoes to a variable voltage with a sinusoidal trend (stress) and the resulting sinusoidal deformation (deformation) is measured and recorded; if the sample is purely elastic, the phase difference “ δ ” between the stress and the deformation is zero (i.e. the sample is in phase), while is the samples if purely viscous “ δ ” will be $\pi/2$. This parameter allows to investigate the material’s response to the application of different forces, such as compression, stress-strain, creep and multi-frequency strain. The DMA is composed of a high resolution linear optical encoder which is able to measure displacements of fractions of a micron and an automatic furnace that efficiently controls the temperature²²⁹.

In this study, the 3D CSCs niche model has been analysed by using the “compression” mode. Briefly, the sample was placed on a fixed flat surface while a rocking plate applied the load. A minimum preload force was set to ensure the optimal contact between the mobile clamp and the sample, obtaining the maximum contact between the surface of the scaffold and that of the mobile part. The compression clamp measures the height of the sample while the diameter must be entered manually²²⁹. For the test, the 3D CSCs niche model, both open and close injection, was developed *in vitro* as reported in paragraph 3.4.2 and culture for 1, 7 and 14 days before the DMA analysis. The scaffold without cells was used as *blank*. The samples presented the size of ~ 9 mm diameter and 6 mm heigh. At each time point, a biological triplicate for open and close injection (N=3) and six biological replicates for *blank* (N=6) were analysed at the DMA. A preload of 0.001 N was applied to each sample and the compression test was performed uniaxially with controlled forced ramp of 0.5 to 5 N at a speed of 0.5 N/min. The obtained stress-strain curve was used to measure the compressive modulus (E) and the slope of the linear fit line was calculated in a strain range from 5 to 11%²⁴⁰.

3.4.2.6. Statistical analysis

Statistical analysis was performed by using GraphPad Prism Software (8.0.1 version). The results of qRT-PCR were plotted in the graph as fold-change expression relative to the control $\pm$ SEM and they were analysed by Two-Way ANOVA and Tukey’s multiple comparisons test²²⁸. The DMA data were plotted in the graph as mean $\pm$ SEM and analysed by Two-Way ANOVA and Dunnett’s multiple comparisons test. *p value ≤ 0.05 , ** p value ≤ 0.01 , ***p value ≤ 0.001 and ****p value ≤ 0.0001 .

3.4.3. *In vivo proof of concept in CAM model*

3.4.3.1. *The development and in vivo grafting of the 3D CSC niche model*

As a conclusive optimization step, the 3D CSCs niche model, both open and close injection, was tested *in vivo* CAM of chick embryo to investigate their ability in inducing the tumour development and growth²²⁸. The 3D CSCs niche model was optimized in terms of size to be engrafted on the CAM of chick embryo at EDD6²⁴¹. Indeed, in order to avoid the membrane collapse, the size and weight (≤ 50 mg) optimization are fundamental to guarantee a successful engrafting. Facing this limitation, starting from the original MgHA/Coll scaffold ($\varnothing$: 9mm; h:6mm), the 3D CSCs niche model was optimized in $\sim \leq 20$ and ≤ 40 mg weight for both open and close injection, respectively, and $\sim \varnothing$:3 mm; h: 3 mm; 1.5 mm thick, by using a combination of sectioning and punching approach.

For the *in vitro* development of the 3D CSCs niche model, the MG63 G1 sarcompheres were obtained by culturing cells in U.L.A. 24 well-plates at a density of 25.000 cells/well (see paragraph 3.4.1.1.) and both close and open injection were obtained as previously reported (see the paragraph 3.4.2.1), using a drop of 10 uL sarcompheres suspension for the injection. After 7 days of culture *in vitro*, both groups, open and close injection, were engrafted *in vivo* CAM at EDD6. The scaffold without cells was used as a control group (CTR).

For the grafting procedure, a small window was opened on the blunt end of the egg at EDD3 to create an air pocket inside it which will allows enough space to work. Tweezers were used to deposit each 3D CSCs niche model on the membrane of the chick embryo at EDD6 after breaking secondary vessels to bring it in contact with the blood system. A total of six biological replicates of open injection (N=6) and a duplicate of both close injection (N=2) and CTR (N=2) were engrafted. The eggs were incubated (FIEM MG 140/200) at 37.5°C/99.5°F and 60% humidity, monitoring the morphological changes and tumor growth from until EDD14. At EDD14, the eggs were incubated for 2 hours at 4°C before harvesting the models and determining their weight. The harvested samples were fixed in PFA 4% or Formalin 10% for 24 hours and processed for further biological analysis.

3.4.3.2. *Tumour monitoring and weight*

The monitoring of the 3D CSCs niche model grafted in CAM models has been performed by using an optical microscope (Dino-Lite USB Microscope) and the DinoCapture 2.0 software²²⁸. At EDD10, 12 and 14 the status of the grafted 3D OS model was checked by images acquisition. The weight of 3D CSCs niche model was determined after the harvesting of the models at EDD14. The data of the tumour weight have been reported in the table as mean (mg) $\pm$ SEM.

3.4.3.3. Histological and Immunohistochemical analysis

The harvested samples were fixed for 24 hours in 10% formalin at 4°C. Then, the samples were embedded in paraffin, sectioned, deparaffined and then hydrated as reported in paragraph 3.4.2.4. before histological staining and immunohistochemistry.

For the immunohistochemical analysis²²⁸, the UltraTek Alk-Phos Anti-Polyvalent (Fast Red) Staining System (Histo-Line Laboratories) was used, following the manufacturer's instructions, using the Methyl Green (Sigma Aldrich) as cell nuclei counterstaining. The sections were incubated with primary antibodies for SOX-2, OCT-4, NANOG, HIF-1 α and NOTCH-1 as reported in paragraph 3.4.2.4. Primary antibodies for CD133 (Prominin-1, 1:250, MA1219, Invitrogen), Ki67 (1:100, MA541135, Invitrogen), PECAM-1 (Platelet and endothelial cell adhesion molecule 1 or cluster of differentiation 31 – CD31, 1:1000, 89C2, Cell Signaling) and vWF (Von Willebrand Factor, 1 μ g/mL, ab201336, Abcam) were performed, following the datasheet instructions. Two biological replicates of open injection (N=2) and one biological replicate of close injection (N=1) were performed.

For the histological analysis²²⁸, the H&E, and Von Kossa (Sigma Aldrich) and Alizarin Red (Sigma Aldrich) for calcium ions and deposits, were performed on the sections, following the manufacturer's instructions, using Nuclear Fast Red was used as counterstaining for cell nuclei.

After the application of the immunohistochemistry kit or histological staining, the sections were mounted by using histology mount as reported in paragraph 3.4.2.4. Images were acquired at the Optical Microscope.

3.4.3.4. Actin and DAPI staining

The morphology of the harvested samples was analysed by Actin and DAPI staining, following the manufacturer's instructions²²⁸. Briefly, the samples were fixed in 4% PFA for 24 hours at 4°C. Then, the samples were embedded in OCT after cryopreservation process as reported in paragraph 3.4.1.7. The sections were obtained and permeabilized as reported in paragraph 3.4.1.7. The sections were then stained with Rhodamine Phalloidin Actin red 555 ready probs for F-actin filaments and DAPI counterstaining. The sections were mounted by Fluor Save reagent and images were acquired at the Inverted Ti-E Fluorescence Microscope using TRITC and DAPI filters.

3.5. The Drug Resistance evaluation of the 3D CSCs niche model

3.5.1. The drug concentration

Once the 3D CSCs niche model has been finalized, the close injection has been selected for the investigation of whether it was suitable to resemble the drug resistance phenomenon of OS both *in vitro* and *in vivo* conditions. For this study, the final size of the 3D CSC niche model has been further defined as reported in paragraph 3.4.3.1. in order to improve the repeatability of the size reduction process and minimize the variability among samples. All the systems were tested with cisplatin,

doxorubicin and dibenzyl diselenide (DBDS). The DBDS compound was kindly provided by Prof. Valerio Voliani of the Center for Nanotechnology Innovation, Istituto Italiano di Tecnologia (IIT), in Pisa, Italy¹¹³.

The chemotherapeutics were used according to the concentrations typically reported in clinic and applying a well-recognized human-animal conversion formula²⁴²; briefly, considering that cisplatin and doxorubicin are typically used at the concentration of 120 and 75 mg/m², the *in vivo* preclinical concentration to be used in CAM has been calculated as follows:

(1) application of the conversion factor²⁴² from clinic to preclinic, considering the average human body weight (60 kg) and surface area (1.62 m²):

$$\left(\frac{X \text{ mg drug}}{m^2}\right) \left(\frac{1.62 \text{ m}^2}{60 \text{ kg}}\right) = Y \text{ mg cisplatin/kg}$$

where X is the drug amount in mg used in clinic per m², and Y is the corresponding drug amount in mg per kg obtained through the formulae.

(2) consideration of the average weight of a single chick embryo at EDD10 (2.3 g)²⁴³:

$$\left(\frac{Y \text{ mg cisplatin}}{\text{kg}}\right) (0.0023 \text{ kg}) = Z \text{ } \mu\text{g cisplatin}$$

where Z is the drug amount in µg to be used on each chick embryo.

An amount of 7.5 and 4.7 µg of cisplatin and doxorubicin, respectively, for each embryo were used. For DBDS, an already validated concentration derived from a previously published work on *in vivo* pancreatic tumour in mice¹¹³, has been selected to be used in this work; briefly, 1 mg/kg DBDS was used to calculate the drug amount for *in vivo* CAM models, considering the weight of the chick embryo at EDD10 as previously reported in the second formula, obtaining a final amount of 2.3 µg of DBDS to be used for each embryo.

The drug solution has been prepared according to 30 µL solution/embryo in DMEM/F-12 GlutaMAX media without any supplements. The media without drug has been used as treatment for the control group (CTR).

For the *in vitro* testing, the drug concentration has been calculated by dividing by 10 (/10) the drug amount used *in vivo* CAM models; since 7.5, 4.7 and 2.3 µg in 30 µL basic media correspond to 830, 270 and 225 µM, respectively, a concentration of 83, 27 and 23 µM for cisplatin, doxorubicin and DBDS, respectively, has been used for the *in vitro* studies.

3.5.2. *In vitro* drug resistance of the 3D CSCs niche model

The MG63 G1 sarcospheres were obtained as previously reported (see paragraph 3.4.1.1.) starting from a density of 50.000 cells/well in U.L.A. 6 well-plates. The 3D CSCs niche model obtained *in vitro* by close injection approach was developed as previously reported (see paragraph 3.4.3.1). After

7 days of growth *in vitro*, the drugs were added to the models at the concentration of 83, 27 and 23 μM for cisplatin, doxorubicin and DBDS, respectively, and the drug-free 3D CSCs niche model was used as control group (CTR). After 24 and 96 hours of incubation with treatments, the 3D CSCs niche model was collected for different biological analysis.

3.5.2.1. Cell viability and proliferation

The cell viability and proliferation of the 3D CSCs niche model was performed at 24 and 96 hours with drugs by Thiazolyl Blue Tetrazolium Bromide (MTT) assay, following the manufacturer's instructions. Briefly, the MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (5 mg/mL) was dissolved in PBS 1X. The 3D CSCs niche model was incubated with the MTT solution at 10% (v/v) concentration for 2 hours at 37°C. Then, the sample was transferred into a centrifuge tube, 600 μL DMSO were added and the 3D CSCs niche model was homogenized by pestle-mediated breaking and vortexing, in order to improve the dissolution of formazan crystals derived from cellular metabolism of the MTT reagent. The sample was incubated in DMSO for 15 minutes at room temperature under slight stirring conditions, followed by centrifugation and the absorbance of the formazan was read in technical triplicate at 570 nm using the Multiskan FC Microplate Photometer (Thermo Fisher Scientific). The reading values are directly proportional to the number of metabolically active cells in each sample. For the study, one experiment was carried out and a biological triplicate for each condition was performed. The sarcospheres-free MgHA/Coll scaffold was used as *blank* to remove the background interference during data analysis.

3.5.2.2. Statistical analysis

Statistical analysis was performed by using GraphPad Prism Software (8.0.1 version). The data of cell viability were plotted in the graph as percentage (%) respect to CTR $\pm$ SEM and analysed by Two-Way ANOVA and Tukey's multiple comparisons test. ****p value ≤ 0.0001 .

3.5.3. *In vivo* drug resistance in CAM models

The MG63 G1 sarcospheres were obtained as previously reported (see paragraph 3.4.1.1.) starting from a density of 50.000 cells/well in U.L.A. 6 well-plates. The 3D CSCs niche model was finalized in terms of size, developed *in vitro* and engrafted *in vivo* CAM of chick embryo as previously reported (see paragraph 3.4.3.1.). After grafting at EDD6, the eggs were incubated until EDD10 to make the tumour develop. At EDD10, the drugs were dripped on the developed tumours at the concentration of 830, 268 and 223 μM for cisplatin, doxorubicin and DBDS, respectively, using the media without drug as CTR (see paragraph 3.5.1.). The tumours were monitored at EDD6, EDD10, EDD12 and EDD14 and a total of 96 hours of treatment incubation has been performed. The sarcospheres-free scaffold was used and grafted as *blank*. The CAM were incubated at 37.5°C/99.5°F and 60%

humidity. At EDD14, the 3D CSCs niche models were harvested as previously reported, weighted and, treated for formalin 10% or PFA fixation and/or store immediately at -80 °C until biological analysis.

3.5.3.1.Finalization of the 3D CSCs niche model's size

Starting from the original MgHA/Coll scaffold (ø: 9mm; h:6mm), the 3D CSCs niche model obtained by close injection approach was further optimized in $\sim \leq 30$ mg weight and $\sim \varnothing:3$ mm; h: 3 mm; 1.5 mm thick, by using a combination of sectioning and punching approach as reported in paragraph 3.4.3.1. The repeatability of the process has been tested by monitoring the weight of the models in media and the cell viability of sarcospheres after injection-based seeding up to 7 days of culture by Live and Dead assay (see paragraph 3.4.2.2. for details on the Live and Dead Assay) (N=10).

3.5.3.2.Tumour monitoring, volume and weight

The monitoring of the 3D CSCs niche models grafted in CAM models has been performed by using an optical microscope and the DinoCapture 2.0 software at EDD6, 10, 12 and 14, as reported in paragraph 3.4.3.2. The volume of the tumour has been calculated measuring the width as shorter diameter and the length as the longer ones by using the DinoCapture 2.0 software and applying a modified ellipsoid formula $\frac{1}{2} \times (\text{length} \times \text{width}^2)^{2/3}$. The weight of the 3D CSCs niche models was determined before and after the grafting in CAM models. For both volume and weight, a total of twenty biological replicates has been performed for each condition (N =20).

3.5.3.3.Gene expression

The gene expression was analysed by Real Time PCR, following the manufacturer's instructions. The harvested samples were separately collected and homogenized in two steps of 500 µL Tri Reagent by pestle-mediated breaking and vigorous vortexing. The RNA extraction and purification, the RNA quantification and RNA reverse transcription into cDNA were performed for each sample separately, following the manufacturer's instructions, as reported in paragraph 3.4.1.6. The Real Time PCR was performed using TaqMan Gene Expression Assay Kits for OCT-4, NANOG, SOX-2, HIF-1 α and IL-6 as reported in paragraph 3.4.1.6 and 3.4.2.3. The CTR without treatment was used as control group and Actin- β was used as housekeeping gene as reported in paragraph 3.4.1.6. Three experiments were performed for a total of four biological replicates for each condition (N = 4). Each biological replicate was analysed in three technical replicates (N=3). Data were collected and elaborated as reported in paragraph 3.4.1.6²³⁹.

3.5.3.4.Histological analysis and semi-quantification of mineralization

The harvested samples were incubated for 24 hours with 10% formalin at 4°C. Then, the samples were embedded in paraffin, sectioned, deparaffined and then hydrated as reported in paragraph

3.4.2.4. before histological staining. The H&E, Von Kossa, Alizarin Red, Alcian Blue and Safranin-O stainings were performed on the sections, following the manufacturer's instructions. The Methyl Green was used as counterstaining for cell nuclei identification in Von Kossa. The Haematoxylin was used as counterstaining in H&E, Alcian Blue and Safranin-O stainings.

After the histological staining, the sections were mounted by using histology mount as reported in paragraph 3.4.2.4. Images were acquired at the Optical Microscope.

The mineralization observed in Von Kossa and Alizarin Red stainings as an indicator of calcium ions deposits was semi-quantified by using ImageJ software²⁴⁵. Briefly, the image was converted to grayscale and the threshold was set to segment the image and define which parts should be included in the analysis based on intensity. The upper and lower bounds of the threshold range were adjusted to highlight the regions to be analysed appearing as red mask; applying the threshold, the image is converted into a binary form with only the selected regions of interest (ROIs). Running the measurement, the Integrated Density represents the total intensity in the selected area, which is calculated as the sum of pixel values. The data was normalized on the CTR and 4 images per group were used for both Alizarin Red and Von Kossa analysis (N=4).

3.5.3.5. Semi-quantitative immunofluorescence analysis

The immunofluorescence detection of specific markers was performed on the sections, following the manufacturer's instructions. Briefly, the samples were fixed in 4% PFA, embedded in OCT and sectioned as reported in paragraph 3.4.1.7. The saturation of the sections was performed in 1% Bovin Serum Albumin (BSA) and 10% Normal Goat Serum (NGS) in PBS 1X for 1 hour at room temperature. Then, the sections were permeabilized in 0.1% Triton-X 100 in PBS 1X for 20 minutes and incubated with primary antibodies for OCT-4 (2 µg/ml), CD133 (1:100), HIF-1α (1:800), Ki67 (1:1000) (see paragraph 3.4.3.3.), Collagen Type IA (1:2000, Ab90395, ABCAM) and CD44 (2 µg/ml, MA5-13890, Thermo scientific).

The Goat anti-rabbit IgG Alexa 546 (1:1000, Molecular Probes) and Alexa Fluor 555 Donkey anti-mouse IgG (H+L) (4 µg/mL, Invitrogen) were used as secondary antibodies, following the manufacturer's instructions. The sections were then stained with Actin green 488 ready probs (Invitrogen) for F-actin filaments detection and DAPI staining was used for cell nuclei counterstaining. The sections were mounted by Fluor Save reagent and images were acquired at the Inverted Ti-E Fluorescence Microscope using TRITC, FITC and DAPI filters to identify positive markers, the cytoskeleton and the cell nuclei, respectively.

The immunofluorescence was semi-quantified by ImageJ software as reported in the paragraph 3.5.3.3²⁴⁵. The data was normalized on the CTR and 4 images per group were measured for the analysis of each antibody (N=4).

3.5.3.6. Statistical analysis

Statistical analysis was performed by using GraphPad Prism Software (8.0.1 version). The data of the tumour volume have been reported in the graph as fold-change of the mean (mm^3) normalized on $\text{EDD6} \pm \text{SEM}$ and they were analysed by Two-Way ANOVA and Tukey's multiple comparisons test. The data of the tumour weight have been reported in the graph as fold-change of the mean (mg) normalized on $\text{EDD6} \pm \text{SEM}$ and they were analysed by multiple t test with significant p value ≤ 0.05 . The results of qRT-PCR are reported in the graphs as fold-change expression relative to the $\text{CTR} \pm \text{SEM}$ and they were analysed by Two-Way ANOVA and Tukey's multiple comparisons test. The results of the semi-quantitative mineralization and immunofluorescence were plotted in the graph as normalized mean respect to CTR and analysed by Two-Way ANOVA and Tukey's multiple comparisons test. *p value ≤ 0.05 , ** p value ≤ 0.01 , ***p value ≤ 0.001 and ****p value ≤ 0.0001 .

3.6. The development of the 3D co-culture model of OS

The 3D co-culture model of OS was developed *in vitro* by combining the 3D MgHA/Coll scaffold with GFP-MNNG HOS osteosarcoma cells and iBM-MSCs. Briefly, the MgHA/Coll scaffold ($\sim \varnothing$:9 mm; h: 6 mm) was pre-conditioned in cell culture media for 24 hours before cell seeding. The GFP-MNNG HOS and iBM-MSCs were mixed at 1:1 ratio and seeded at the total density of 50.000 cells/scaffold. The model was incubated for 20 minutes for cellular pre-adhesion before cell media addition. Both cell types were seeded at a density of 50.000 cells/scaffold to obtain the mono-culture conditions. The same conditions were performed under scaffold-free conditions. After 7 and 14 days of culture, the models were collected for the different biological analysis.

3.6.1. Cellular colonization and Actin and DAPI staining

The cell morphology of the 3D co-culture model was analysed by Actin and DAPI staining, following the manufacturer's instructions. The samples were fixed in 4% PFA and permeabilized in 0.1% Triton-X 100 in PBS 1X, following the manufacturer's instructions. The samples were stained with Rhodamine Phalloidin Actin red 555 ready probs for F-actin filaments detection and DAPI staining was used for cell nuclei counterstaining.

For the cellular colonization, the scaffolds at day 7 were vertically sectioned by using a scalpel and the distance travelled by the cells from the seeding surface to the core of the scaffold has been calculated using the NIS-Element software for image analysis (Nikon) and the "line" tool. For the analysis, 3 images/condition were acquired and 4 measurements/image were performed and mediated. For the experiment, two biological replicates for each condition were performed ($N = 2$) and the images were acquired at the Inverted Ti-E Fluorescence Microscope using TRITC, FITC and DAPI filters for MSCs, GFP MNNG-HOS cells and cell nuclei identification, respectively.

3.6.2. Scanning Electron Microscopy (SEM)

The cell morphology of 3D co-culture model was also analysed by SEM (ESEM Quanta 200, FEI, Thermo Fisher Scientific Inc.), following the manufacturer's instructions. Briefly, the samples were fixed in 2.5 % (v/v) Glutaraldehyde (Merk KGaA) in 0.1 M (w/v) Sodium Cacodylate Buffer (pH 7.4, sigma) for two hours at 4°C. For the SEM analysis, the samples were washed twice in water for 10 minutes and frozen at -20°C overnight before being freeze-dried (– 40 °C and + 25 °C) for 48 hours under 0.086 mbar vacuum conditions (5 Pa, LIO 3000 PLT). Then, the samples were fixed on aluminium stubs by using adhesive tape and graphite glue, and sputtered coated with 20 nm thin gold film. The images were acquired under high vacuum conditions. For each condition, a biological duplicate was performed (N=2).

3.6.3. Gene expression

The gene expression was analysed by Real Time PCR, following the manufacturer's instructions. Each 3D sample was separately collected and homogenized in two steps of 300 µL of Tri Reagent by pestle-mediated breaking and vigorous vortexing at each defined time point. For scaffold-free samples, 1 mL of Tri Reagent/well was used to homogenize the cells. The RNA extraction and purification, RNA quantification and the RNA reverse transcription in cDNA were performed as reported in paragraph 3.4.1.6. During the RNA extraction and purification, the samples were combined two by two into a single Zymo-Spin IICR Column to increase the RNA yields.

The Real Time PCR was performed using TaqMan Gene Expression Assay Kits for NANOG (see paragraph 3.4.1.6.), CD105 (Endoglin, Hs00923996_m1), CD90 (Thy-1, Hs00264235_s1) and CD73 (NT5E, Hs04234686_m1) to investigate the stem and mesenchymal phenotype of MSCs. TaqMan Gene Expression Assay kits for ALP (ALPL, Hs01029144_m1), BMP-2 (Hs00154192_m1) and RUNX-2 (Hs00231692_m1) were used to investigate the potential osteogenic commitment of MSCs in the co-culture system, together with HIF-1 α (see paragraph 3.4.2.3.), IL-6 (see paragraph 3.4.2.3.), IL-8 (Hs00174103_m1) and ACTA2 (Hs00426835_m1) as markers of the tumour microenvironment and the potential MSCs differentiation toward CAFs.

Different types of analysis were performed; the MSCs mono-culture under both 3D scaffold-based and 2D conditions was used as control group as well as the 2D conditions compared to 3D ones. Actin- β (see paragraph 3.4.1.6.) and GAPDH (Hs99999905_m1) were used as housekeeping gene. Two experiments were performed with a total of six biological replicates for each condition (N = 6). Each biological replicate was analysed in three technical replicates (N=3). Data were collected and elaborated as reported in paragraph 3.4.1.6²³⁹.

3.6.4. ELISA kits

The detection of MMP-2, MMP-9, Tissue Inhibitor of Metalloproteases – 3 (TIMP-3) and TIMP-4 has been performed by a solid-phase sandwich Enzyme-Linked Immunosorbent Assay (ELISA) (Invitrogen) designed to detect and quantify the level of antigen in cell culture supernatants. Specifically, the 3D co-culture models were developed *in vitro* as reported in paragraph 3.6 and after 7 and 14 days the supernatant was collected, centrifuge at 2000 xg for 20 minutes at 4°C to remove cellular debris and stocked at -80°C until the analysis. The ELISA Kits (Invitrogen) were performed following the manufacturer's instructions. The background absorbance was subtracted from all data points, including standards and controls, before to interpolate the data on the standard curve. The standard curve was generated by using the curve-fitting mode of the GraphPad Prism software (version 8.0.1).

3.6.5. Statistical analysis

Statistical analysis was performed by using GraphPad Prism Software (8.0.1 version). Different types of analysis were performed for the qRT-PCR; the results were plotted in the graphs as fold-change expression relative to the MSCs mono-culture under both 3D scaffold-based and 2D conditions as well as to the 2D conditions compared to 3D ones, $\pm$ SEM and they were analysed by Two-Way ANOVA and Sidak's multiple comparisons test. The results of ELISA were plotted in the graphs as mean $\pm$ SEM and analysed by Two-Way ANOVA and Tukey's multiple comparisons test. The cell colonization data was plotted in the graph as mean (μ m) $\pm$ Standard Deviation and analysed by One-Way ANOVA and Tukey's multiple comparisons test. *p value $\leq$ 0.05, ** p value $\leq$ 0.01, ***p value $\leq$ 0.001 and ****p value $\leq$ 0.0001.

4. RESULTS AND DISCUSSION

4.1. The characterization of the MgHA/Coll scaffold

The MgHA/Coll scaffold as well-established²²³ and used bone-like matrix for regenerative medicine applications²²², has been synthesized and characterized by the ISSMC of CNR of Faenza, and used in this study. The MgHA/Coll scaffold is a biomaterial which is synthesized through a naturally bone formation-inspired process, to overcome the limitations of other matrices typically proposed for tissue engineering and regenerative medicine purposes. Naturally inspired biomaterials ensure full biocompatibility thanks to a cell-friendly porosity with appropriate interconnection and size, improving cellular colonization and avoiding challenges associated with the cellular behavior impairment induced by synthetic materials^{246, 247}. Moreover, the environmental signals provided by these biomaterials to the cells are more predictive of the *in vivo* signalling pathways due to their naturally-inspired synthesis. Considering the determinant role of the environmental signals for the cellular behavior and the reliability of the results²⁴⁸, and the increasingly relevant challenge in mimic the TME by the chemical, physical, biochemical and architectural points of view, the MgHA/Coll scaffold has been selected for the studies²²⁸.

The naturally-inspired synthesis of MgHA/Coll scaffold is based on the neutralization reaction between a basic suspension of calcium hydroxide added with magnesium chloride and an acid suspension of phosphoric acid with type I collagen. It is precisely this pH variation, from 10 to 6, which induces the simultaneous precipitation of the mineral phase nanocrystals (MgHA) and the self-assembling of collagen fibers (Coll), enabling the reproduction of the bone formation process and the creation of a hybrid material with chemical features similar to that one of the natural bone matrix²⁴⁹. After the synthesis, the MgHA/Coll scaffold has been deeply characterized in terms of morphological, chemical structure swelling and degradation behaviour (Figure 4)²²⁸.

The SEM²²⁸ was used to investigate the 3D morphology and structure of the scaffold (Figure 4A). The results showed that the material presents an isotropic structure with randomly distributed and interconnected macro- and micro-porosity, thanks to the freeze-drying process on the obtained hydrogel helping to improve the stability and the interconnection of the structure and, consequently, the cellular colonization ability. The total porosity of the scaffold was assessed at ~ 80% and it depended on the amount of water present in the hydrogel during the freeze-drying process (Figure 4B). Most important, it is possible to observe at higher magnification the full embedding and homogenous distribution of MgHA nanoparticles on the collagen fiber, giving rise to a self-assembled collagen mineralized fiber as a typical feature of the bone mineralization process (Figure 4A).

The ICP-OES²²⁸ analysis showed that the MgHA/Coll scaffold contains a Mg/Ca ratio of 0.05 and (Mg+Ca)/P molar ratio of 1.63, compared to the Ca/P = 1.67 of stoichiometric apatites, assessing the

presence of a substituted and poorly crystalline apatite (Figure 4B), which is a highly biomimetic mineral phase. This was also confirmed by the broad diffraction peaks of XRD pattern²²⁸, pointing out the presence of a pure hydroxyapatite phase with low crystallinity and the absence of secondary phases (Figure 4D). The presence of Mg^{2+} ions is determinant for this result, and they are typically presented in the ECM during the biomineralization of the bone; during the process, the functional groups of the organic matrix act as sites for the nucleation of the mineral phase through the precipitation of ions, including Mg^{2+} ²⁵⁰. In this context, the mineral phase grows under strict physical constraints which restrict the crystal size, morphology and solubility. In the MgHA/Coll scaffold, the calcium substitution with Mg^{2+} ions increase the hydroxyapatite nucleation but inhibits its crystallization reducing the Ca/P ratio^{223, 240}.

The TGA analysis²²⁸ assessed the mineral phase content in the scaffold, reporting a 44:56 ratio of Coll to MgHA (Figure 4C). Indeed, the TGA curve reported three stages of weight loss: (1) the first one occurred between 25°C and 160°C and it is related to the release of absorbed and bonded water; (2) the second stage happened between 160°C and 360°C due to the degradation of Type I collagen; (3) the last stage was related to the combustion of organic residues between 360°C and 660°C. The residual weight corresponded to the content of the mineral phase (Figure 4C).

The FTIR spectra²²⁸ confirmed the chemical structure and bonds present in the material, showing the typical peaks of amides (II, II, III) stretching and bending vibrations at 1640, 1545 and 1236 cm^{-1} , respectively, corresponding to the alpha-helical structure of collagen (Figure 4E). The peaks of phosphate ion PO_4^{3-} (474, 569, 602, 962, 1045 and 1091 cm^{-1}) and OH^- (633 and 3572 cm^{-1}) groups were showed by the spectra as typical hydroxyapatite peaks. The ester bonds induced by DHT treatment were shown by the shoulder at 1713 cm^{-1} , while the chemical interaction between the mineral phase and the collagen was confirmed by the shift from 1340 cm^{-1} to 1337 cm^{-1} , which is caused by the chemical bond between the carboxylic groups of collagens and the Ca^{2+} ions of apatite. The bands at 3497 and 1638 cm^{-1} indicated the presence of lattice water in the material (Figure 4E). Finally, the immersion-based method was used to assess the stability of the cross-linking and swelling behavior in physiological conditions (37°C and PBS 1X) (Figure 4F and G)²²⁸. The results reported a hydrophilic behavior by the scaffold with a water uptake of three times its weight (Figure 4F), together with a low degradability showing less than 2 wt.% degradation in 28 days (Figure 4G), indicating the successful improvement of scaffold stability by DHT treatment and assessing the MgHA/Coll scaffold as suitable for cell culturing.

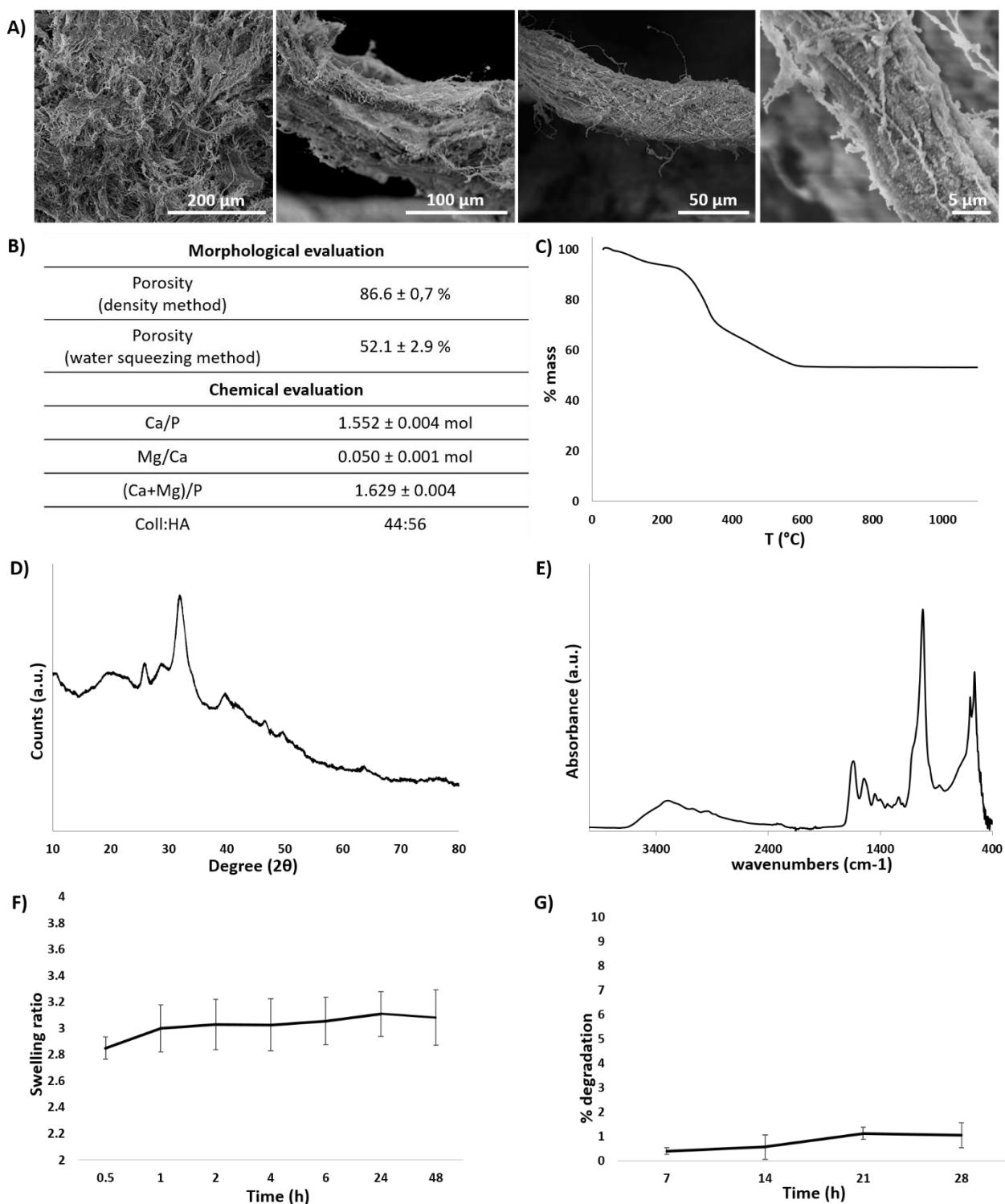


Figure 4. Characterization of the 3D MgHA/Coll scaffold²²⁸. (A) SEM images (N = 3); (B) Total and macro-porosity and chemical evaluation by ICP-EOS (N = 8); (C) Thermal decomposition profile by TGA: the weight loss in % mass in function of the temperature ($^{\circ}\text{C}$) is used to determine the organic/inorganic ratio (N = 8); (D) XRD pattern (N = 8); (E) FTIR profile (N = 8); (F) Time-dependent swelling behaviour: time after soaking in PBS 1X on X-axis (N = 8); (G) degradation profile after 7, 14, 21, 28 days in PBS 1X (N = 8).

4.2. The 3D CSCs niche model

The osteosarcoma is a highly malignant tumour which remains fatal for one-third of drug-resistant patients due to the lack of effective therapies. This challenge is exacerbated by a limited understanding of the disease's complex biology²⁵¹ due to its heterogeneity and instability to which the CSCs contribute⁸.

To overcome these challenges, the development and use of more predictive *in vitro* systems that accurately mimic tumour complexity is well-recognized to improve the biological exploration of the OS and the reliability of drug screening results. In this study, two well-established tumor-engineering approaches—the cellular spheroids and 3D bone-mimetic scaffolds—were employed to develop an *in vitro* 3D Osteosarcoma Model (3D 3D OS model) specifically focused on the Cancer Stem Cells (CSCs) niche. These 3D models were designed to more effectively induce CSC-specific characteristics compared to spheroid-based models alone, offering a closer replication of the Tumour Microenvironment (TME). The study involves two optimization steps: (1) the *in vitro* biological characterization of serial sarcospheres and (2) the optimization of their niche's structural configuration inside the 3D MgHA/Coll bone-like scaffold using custom-designed approaches.

4.2.1. The characterization of Serial Sarcospheres Generations

The sarcospheres-forming culture is a well-known protocol in literature to enrich stem-like cells from tumour cell lines and solid tumours in a spheroid phenotype²⁵². In addition, the serial passaging of sarcospheres has been recently reported as a simple and effective approach improving the enrichment of stem and tumoral features in spheroids due to the simulation of growth regulation similar to solid tumours²⁵³. Different cell lines can form spheres with distinctive morphological and functional features, but OS cells have not been explored so far in this sense²⁵⁴.

In this study²²⁸, four Sarcospheres Generations (Gs) of both MG63 and SAOS-2 osteosarcoma cell lines, here named G0, G1, G2 and G3, were serially developed *in vitro* and biologically characterized with the final aim of selecting the generation expressing the highest tumoral and stem properties to be used for the development of the 3D CSC niche models. Each generation has been analysed regarding cell proliferation and Sphere-Forming Efficiency (SFE) up to 10 days of culture, showing significantly different cell behaviour (Figure 5).

MG63 Gs showed a cell proliferation peak after 3 days of culture (Figure 5A) with a significant difference in G1, G2 and G3 compared to day 1 ($p \text{ value} \leq 0.0001$). G1, G2 and G3 showed an overall higher proliferative potential compared to G0, while G2 and G3 were proliferative compared to G1 (see Figure 5A1 for specific p values), demonstrating a correlation between passage number and proliferation gradually increasing across serial passages, confirming the literature^{255, 256}.

At day 7, a significant decrease in cell growth was reported in all Gs compared to day 3 (p value ≤ 0.0001 and p value ≤ 0.01) except for G1, which displayed the most stable proliferation trend (Figure 5A). Specifically, after an increase at day 3, MG63 G1 proliferation reached a plateau with a significant number of live cells compared to G0 and G2 up to day 10 (see Figure 5A1 for specific p values). A similar proliferation trend has been observed in SAOS-2 Gs (Figure 5B) on day 3, where G1 and G2 showed a significant proliferation compared to day 1 (p value ≤ 0.001 and p value ≤ 0.0001). Despite the significantly higher number of live cells in G1, G2 and G3 compared to G0 up to day 10 (see Figure 5B1 for specific p values), the proliferation ability of all Gs significantly decreased over time (p value ≤ 0.0001) (Figure 5B). The progressive decrease of proliferation trend observed in both cell lines may be potentially due to the formation of a necrotic core within sarcospheres as seen in the typical layered structure of tumour spheroids²⁵⁵. Indeed, studies reported that the size of the necrotic core enhances over time, while the number of proliferating cells decreases, confirming the collapsing proliferation trend of our results^{255, 257}. This trend was notably pronounced in SAOS-2 cells, while the stable trend of MG63 G1 may indicate the preserved proliferative capacity and stability between the necrotic core and surrounding live cells for up to 10 days.

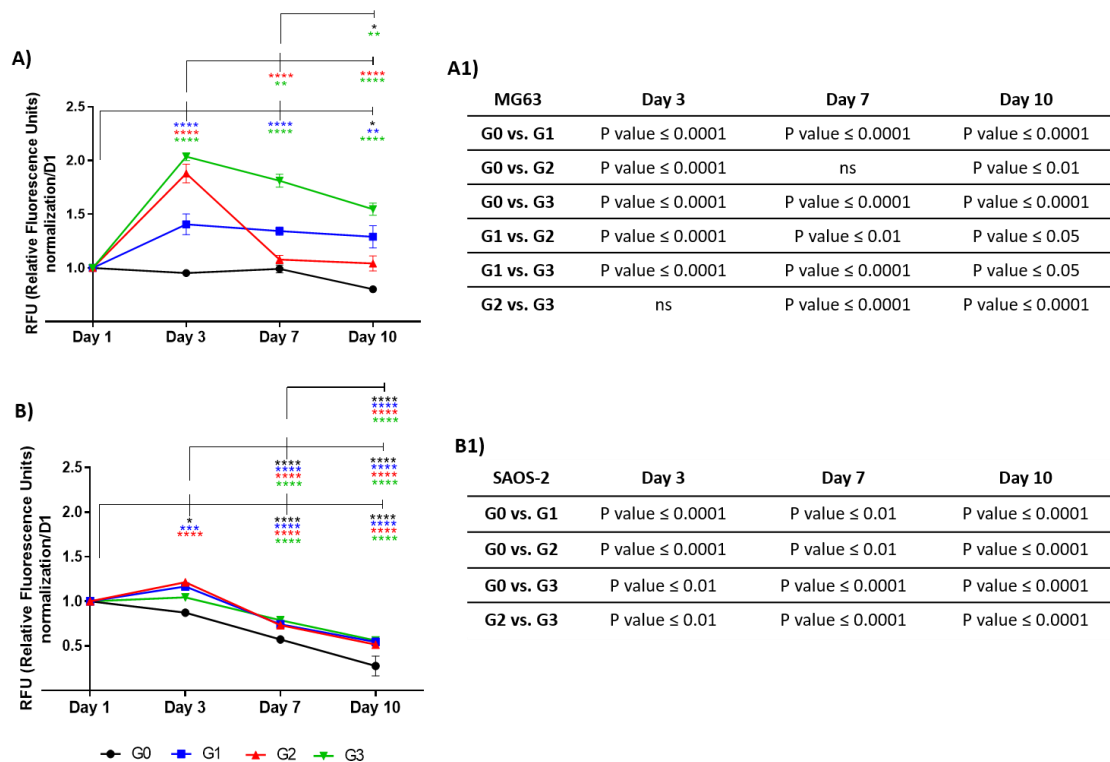


Figure 5. Proliferation of MG63 and SAOS-2 Gs²²⁸. Presto Blue Cell Viability Reagent for MG63 Gs (A) and SAOS-2 Gs (B) as mean $\pm$ SEM (N=6). Significant differences over time are graphically reported, while significances among Gs are reported in the tables for MG63 (A1) and SAOS-2 (B1), respectively. * p value ≤ 0.05 , ** p value ≤ 0.01 , *** p value ≤ 0.001 , **** p value ≤ 0.0001

The SFE indicates the percentage of cells within a cell culture that can form a sphere from a single cell. Since this property is only attributed to stem-like cells, the SFE represents a valuable qualitative and quantitative assay based on exclusively functional features of cells, facing the still challenging identification of CSCs due to the lack of specific and universally applicable markers²⁵⁸. In this study²²⁸, the SFE and size distribution were assessed according to a defined dimensional range (50-99 μm , 100-199 μm , ≥ 200 μm diameter), reporting overall higher SFE values and diameter size in MG63 Gs, compared to SAOS-2 (Figure 6A, B, C, D, E, F). Relevant is the well-preserved round-shape spheroidal phenotype in MG63 Gs during serial passaging, compared to SAOS-2 (Figure 6G) as a well-recognized crucial marker for self-renewing cells within the tumour tissue²²⁸. Conversely, SAOS-2 cells, despite the re-formation of spheres after each dissociation passage, exhibited irregular and unstable morphology, suggesting the potential loss of stem-like cells²⁵⁹. Looking in detail at the Gs, it is clear that G2 showed the most marked increase of SFE in both cell lines, suggesting a potential directly proportional correlation between the proliferation grade and the SFE as an efficient indicator of the presence of cell subpopulation able to form a sphere²⁵⁸; in MG63, G2 reported a SFE value of $6.20\% \pm 0.81$ compared to $3.77\% \pm 0.58$, $3.22\% \pm 0.38$ and $5.45\% \pm 0.74$ of G0, G1 and G3, respectively, with a significant difference between G2 and G1 (p value ≤ 0.05) (Figure 6A). SAOS-2 Gs showed a significant increase in G2 respect to G0, G1 and G3; indeed, G2 showed a SFE value of $1.56\% \pm 0.24$ compared to $0.71\% \pm 0.07$, $0.87\% \pm 0.12$ and $0.83\% \pm 0.18$ of G0, G1 and G3, respectively, with a significant difference compared to G1 and G3 (p value ≤ 0.05) and to G0 (p value ≤ 0.01) (Figure 6D). However, MG63 cells overall formed 3-4 times more sarcospheres per seeded cells than SAOS-2, suggesting varied capabilities among osteosarcoma cell lines in forming sarcospheres due to the intra-tumoral complexity²⁶⁰.

The size distribution showed a different tendency in the two cell lines (Figure 6B, E). The $55.18\% \pm 3.62$ of MG63 G1 reached a 100-199 μm diameter range, with a significant difference with respect to G2 and G3 (p value ≤ 0.001 and p value ≤ 0.01) (Figure 6B), and to the $29.28\% \pm 4.02$ of G1 with diameter ranging in 50-99 μm (p value ≤ 0.0001) (Figure 6B1). The majority of MG63 G0 showed a 100-199 μm diameter range ($48.19\% \pm 2.23$), while G2 and G3 form sarcospheres with a lower diameter ranging in 50-99 μm ($57.70\% \pm 2.74$ and $50.01\% \pm 4.47$ for G2 and G3) with a significant difference compared to G1 (p value ≤ 0.0001) (Figure 6B). Interestingly, MG63 G1 formed a high number of sarcospheres with a diameter ≥ 200 μm , with a significant $15.53\% \pm 1.97$ with respect to $4.98\% \pm 1.12$ and $2.85\% \pm 0.61$ of G0 (p value ≤ 0.05) and G2 (p value ≤ 0.01) (Figure 6B). The largest size distribution of MG63 G1 confirmed the active cell behaviour and stable proliferation trend²⁶¹ and these elements may imply a greater number of self-renewing cells within the sarcospheres as suggested by Neves et al²⁶². Differently, the diameter of SAOS-2 sarcospheres was lower compared

to MG63, with a higher % in the range of 50-99 μm in all Gs; the higher % of sarscospheres in the diameter range of 100-199 μm was shown by G0 and G2 reporting significant differences compared to other Gs (P value ≤ 0.0001) (Figure 6E and 6E1).

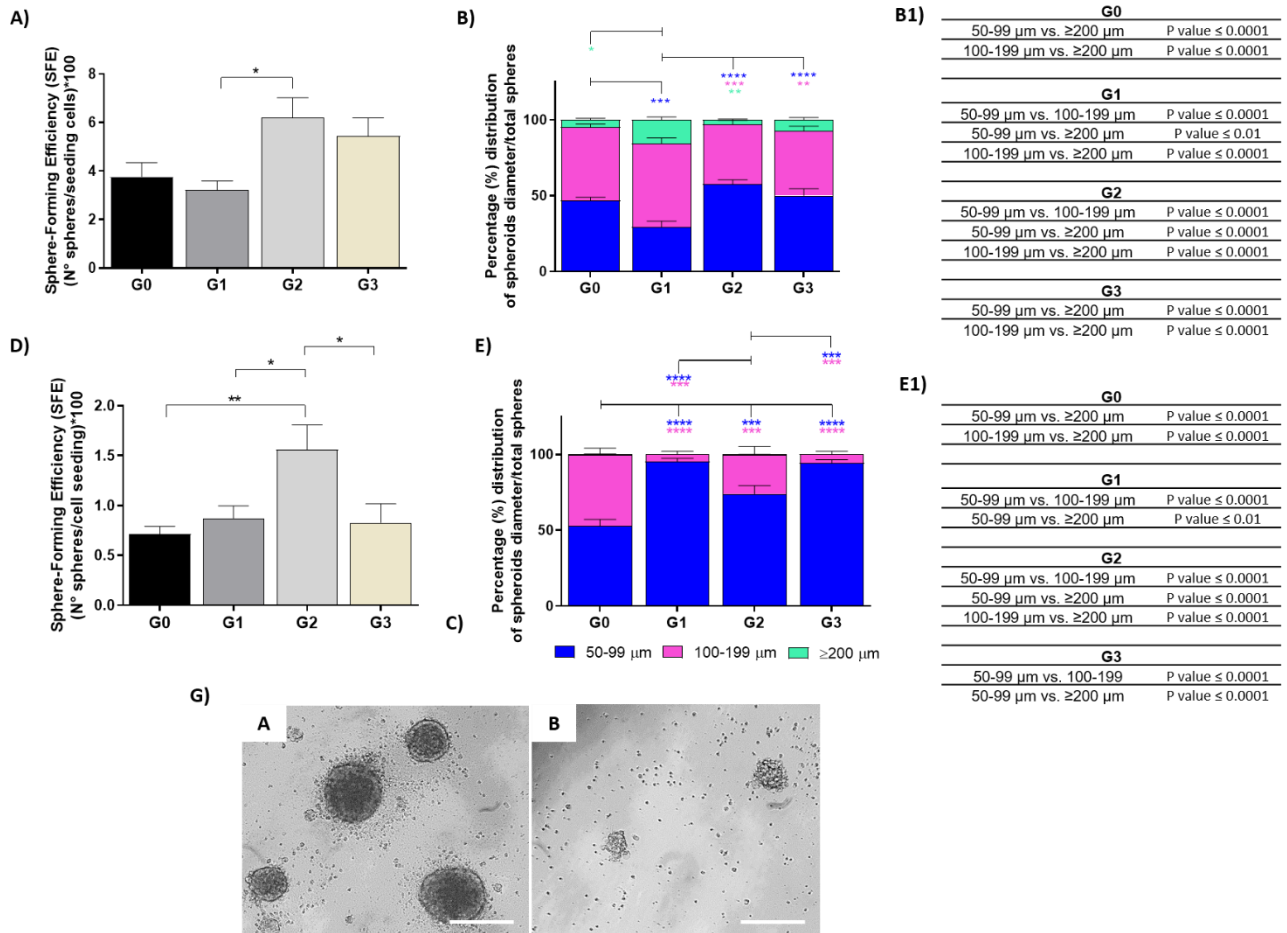


Figure 6. SFE and size distribution of MG63 and SAOS-2 Gs²²⁸. SFE for MG63 Gs (A) and SAOS-2 Gs (D) is reported in the graphs as total % SFE (N=3); the size distribution of MG63 Gs (B) and SAOS-2 Gs (E) is reported as % distribution of spheroids diameter/total spheres (N=3). For the size distribution, significant differences among Gs are graphically reported, while significant differences among dimensional ranges in each Gs are reported in the tables for MG63 (B1) and SAOS-2 (E1). (G) Representative panel of the spheroidal phenotype of Gs in MG63 (A) and SAOS-2 (B). * p value ≤ 0.05 , ** p value ≤ 0.01 , *** p value ≤ 0.001 , **** p value ≤ 0.0001 .

Given the instability of SAOS-2 Gs, a more detailed evaluation of the SCF, migration, invasiveness, gene expression and cell morphology was performed in MG63 Gs in this study²²⁸ (Figure 7). The SCF has been calculated by performing the Limiting Dilution Assay (LDA) and using the Extreme Limiting Dilution Assay (ELDA) software as reported in paragraph 3.4.1.1.3. The LDA has long been used to study stem cells and it is employed to evaluate tumorigenicity in subpopulations of cells as the term limiting dilution derives from the assay's readout, which identifies the lower limit for the number of cells that are required for tumorigenicity. The presence of stem-like cells in a tumour cell

population can be identified by LDA relying on the premise that only CSCs can undergo asymmetrical division to generate the heterogeneous tumour²⁶³. In addition, the ELDA is a software application for LDA, with particular attention to the needs of stem cell assays. It represents the first limiting dilution analysis software to provide meaningful confidence intervals for all LDA data sets, including those with 0% or 100% responses²³⁸. Different statistical analysis is provided by the software, such as the test of the adequacy of the single-hit hypothesis, and that one for the frequency differences between multiple data sets. After ELDA application, the result is expressed as confidence intervals for 1/(stem cell frequency). In this study²²⁸, the SCF as frequency of 1 stem cell plotted with 95% confidence interval was found to be highly variable among Gs (Figure 7A, 7A1). G1 estimated the lowest confidence interval with a frequency of 1 stem cell every 14.6 cells (1:14.6), compared to 1:22.2, 1:80.2 and 1:29.8 for G0, G2 and G3 (Figure 7A1), with a significant difference with respect to G2 (p value ≤ 0.000118); the lowest SCF of MG63 G1 indicates the presence of a higher number of self-renewing cells and stable proliferation trends, making it the preferred model among Gs. G0 and G4 also reported a significant frequency compared to G2 (Figure 7A3). The test for differences in stem cell frequency between all the generations reported a significant Chi-Square as an overall discrepancy in SCF (Figure 7A2).

The migration and invasion ability, as main manifestations of tumour biology and critical components of the metastatic process²⁶⁴, were investigated in this study²²⁸. The migration is the direct movement of cells on a substrate such as basal membrane, ECM or fibers, occurring on 2D surfaces without any obstructive fiber network. The invasion is the cell movement through a 3D ECM²⁶⁴. In this study, the transwell-based method was used since it allows the migration analysis by involving the chemotaxis of cells through a porous membrane after the establishment of a chemoattractant gradient through two medium-filled compartments. In addition, the incorporation of an ECM on the top of the porous membrane permits chemotaxis of cells which possess invasive properties²⁶⁵. The migration and invasion analysis confirmed that G1 has the generation with the highest migratory and invasive potential (Figure 7B, C, D). G1 showed a significantly higher number of migrating cells with respect to G0 and G2 (p value ≤ 0.01 and p value ≤ 0.001) (Figure 7B, D), and invading cells with respect to G0, G2, and G3 (p value ≤ 0.0001) (Figure 7C, D).

The gene expression profile of pluripotency-associated markers of CSCs was also investigated²²⁸, reporting an upregulation in G1 for SOX-2 (~2.18 fold-change) and also for NANOG (~1.79 fold-change), even without significant difference compared to the other Gs. A significant expression of SOX-2 was observed in G3 (~6.40 fold-change) compared to other Gs (p value ≤ 0.0001), while OCT-4 and NANOG did not show significant changes compared to G0 (Figure 7E). The G1 morphology

reported again the preservation of the spheroidal phenotype with healthy cell nuclei and actin filaments (Figure 7F).

Overall, the biological characterization of Gs allowed the clear identification of MG63 G1 as the selected model of enriched-CSCs for the implementation of the CSCs niche in the 3D MgHA/Coll scaffold.

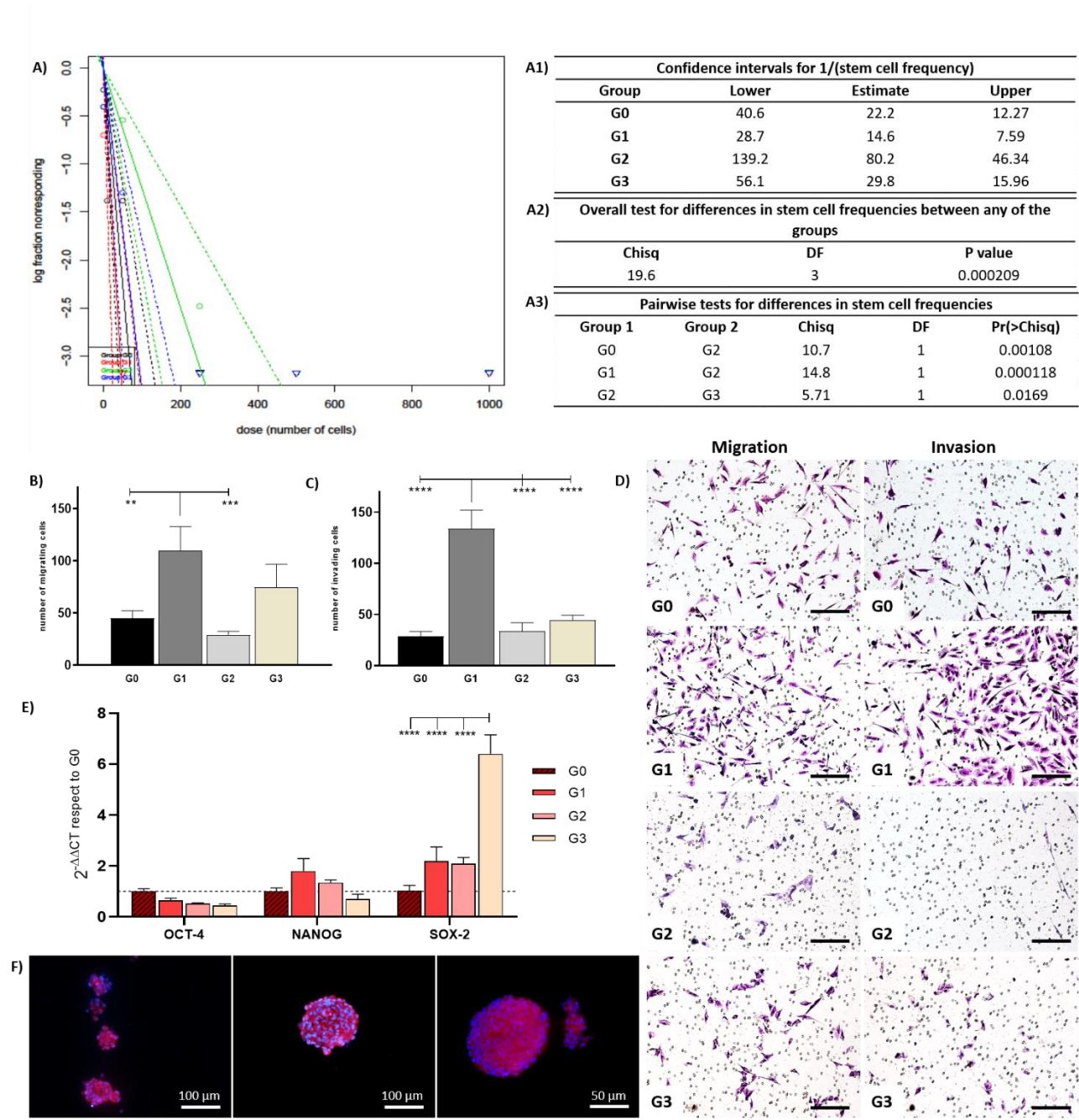


Figure 7. SCF, Migration and Invasion ability, gene expression and cell morphology of MG63 Gs²²⁸. (A, A1, A2, A3) SCF (N= 16): (A) scatter plot with the corresponding trend line of linear regression with 95% confidence interval in dotted lines and data value with zero negative response in the down-pointed triangle; (A1) frequency intervals for 1 stem cell; (A2) significant differences between any of the generations; (A3) significant differences between coupled generations. (B) Cell migration and (C) invasion reported as mean of counted cells $\pm$ SEM (N=2) (D) Crystal Violet

staining of migrating and invasive cells in the membrane of transwell inserts. Scale bars 200 μm . (E) qRT-PCR of stem markers as fold-change expression relative to G0 $\pm$ SEM (N=3). (F) Actin and DAPI staining of G1. F-actin filaments in red, cell nuclei in blue (N=1). * p value ≤ 0.05 , *** p value ≤ 0.001 , **** p value ≤ 0.0001 .

4.2.2. The evaluation of the *in vitro* 3D CSCs niche model

Based on the characterization of Gs, the MG63 G1 was selected to implement the 3D CSCs niche model²²⁸. It is well known that CSCs require environmental signals to survive and preserve their properties. In a previous study²²², a standard seeding of sarsospheres concerning the dripping of the cellular suspension on the material's surface was performed and different 3D OS models have been reported exploiting this simple and shallow cell seeding approach; however, it is well-recognized that CSCs live in a distinct and close anatomical niche of the tumour mass in contact with defined environmental conditions (i.e. hypoxia, low pH) in all directions²⁶⁶. For this reason, the use of the standard seeding is not appropriate and effective to study CSCs, as the absence of a defined confinement in a region which is able to induce a mimetic biochemical environment in terms of nutrients restriction, low oxygen and biomolecules is not possible using a superficial cell culturing²⁶⁷. Recent technologies (e.g. bioprinting) allow cellular embedding into a matrix, however, the difficult bioink selection makes them less suitable for specific applications²⁶⁸. In different studies, natural (i.e. collagen, silk fibroin etc.) or synthetic materials (i.e. polyethylene glycol, poly-lactico-glycolic acid etc.) are employed as matrix²⁶⁸⁻²⁷⁰, but most of them are not bone-inspired materials, in the OS context in which bone composition profoundly affects tumor onset²⁷¹.

Considering this, an injection-based approach for enriched-CSCs seeding into the MgHA/Coll matrix of the scaffold was designed, optimized and analysed in this study²²⁸.

The MG63 G1 were injected with 20 μL of cell suspension/scaffolds in the core of the MgHA/Coll scaffold (Figure 8) with the final aim to address the limitations reported by standard superficial seeding on scaffolds. A live and dead assay was preliminarily performed showing that the sarsospheres were able to successfully grow and colonize the injection hole preserving a high number of live cells and their spheroidal phenotype until 14 days of culture (Figure 8A, B, C).

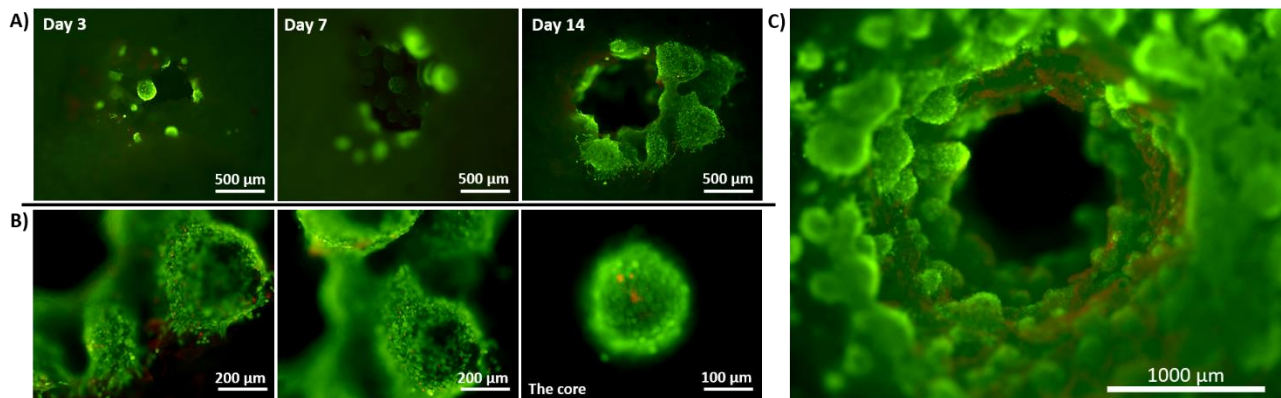


Figure 8. Preliminary evaluation of 3D injection-based cell culture by Live and Dead assay²²⁸. (A) Growth of MG63 G1 inside the injection hole up to day 14; (B) spheroidal phenotype of G1 at 14 days of culture; (C) Representative image of G1 colonization of the injection hole (N=1). Live cells in green, dead cells in red.

Considering the promising preliminary results, the approach has been further optimized developing two different injection-based methods for G1 seeding in the MgHA/Coll that were deeply investigated: close injection and open injection. For the close injection, after G1 injection in the core of the scaffold, wet flakes of MgHA/Cll material were used as a cap to close the injection-derived scaffold hole, while no caps were used for the injectionone. Through these approaches, 3D tumour environments where G1 is in contact with the surroundings in all directions have been developed and the additional flakes of the close injection specifically contribute to the development of a completely close microenvironment at 360°C, with the final aim to resemble the environmental conditions of the tumour niche.

In this study²²⁸, both groups of 3D CSCs niche model were analysed after 7 days of culture, in terms of gene and protein expression, morphological analysis and mechanical properties.

The gene expression profile showed the upregulation of stem markers (OCT-4, NANOG, SOX-2) in both 3D CSCs niche models, compared to scaffold-free G1 as control (Figure 9A), assessing the relevant role of the 3D MgHA/Coll scaffold and the injection-based seeding approach in increasing the stemness phenotype of the sarcospheres²⁷². Indeed, OCT-4, NANOG and SOX-2 are transcriptional factors that are responsible for the maintenance of the self-renewing ability and pluripotency of sarcospheres, and their expression is strictly related to the preservation of the spheroidal phenotype and proliferative profile observed. Specifically, a significant expression of OCT-4 (~5.068 fold-change) (p value ≤ 0.001), NANOG (~5.263 fold-change) (p value ≤ 0.0001) and SOX-2 (~5.798 fold-change) (p value ≤ 0.0001) was observed in the close injection, while only a significant modulation of NANOG (~3.753 fold-change) (p value ≤ 0.01) was reported by the open injection compared to the control (Figure 9A). It's important to notice that the close injection significantly expressed OCT-4 (p value ≤ 0.05) and SOX-2 (p value ≤ 0.01) compared to the open injection (Figure 9A), suggesting that the close microenvironment has a major impact on stem properties. This upregulation trend was confirmed by the positivity of the immunohistochemistry analysis for stem markers in the close injection (Figure 9C).

The expression of CSC niche-related markers was also investigated to understand how the the bone mimetic matrix and the injection-based approach affect the TME. Hypoxia-related marker HIF-1 α was significantly higher in the close injection (~ 1.961 fold-change) with respect to the control (p value ≤ 0.05) and compared to the open injection (p value ≤ 0.05) (Figure 9B). An upregulation trend of the CSC niche-related marker IL-6 (~1.504 fold-change) was also observed in the close injection

group (Figure 9B) even if with no statistically significant difference. These results further highlight the role of the microenvironment of the close injection in triggering the CSC phenotype²⁷³ and inducing hypoxic conditions, crucial for the increase of stem features in cancer cells²⁷⁴. Indeed, HIF-1 α , highly induced in hypoxic conditions, stimulate stem features regulating CSCs generation, maintenance and survival²⁷⁵, while IL-6 is a pleiotropic cytokine involved in maintaining stem properties²⁷⁶ and the upregulation of HIF-1 α , further enhancing CSCs phenotype²⁷³. NOTCH-1 deregulation was shown by both 3D CSCs niche models and it may be attributed to its dual role in tumorigenesis, depending on the tissue type, genetic alterations and receptor-ligand interactions²⁷³. However, no relevant differences in the protein expression of these markers were reported by immunolocalization assay in both 3D CSCs niche models (Figure 9C). The gene expression results showed that the scaffold demonstrated cell-instructive performance, confirming its mimesis with the bone and, most importantly, it is possible to assert that in the 3D CSCs niche model the cell's confinement in a defined region of the matrix allows more the recapitulation of CSCs *in vivo* conditions, with particular attention to the close injection approach.

The morphology of the 3D CSCs niche model was also investigated by Actin and DAPI and H&E staining, showing no relevant differences between open and close injection (Figure 9D, E); in both groups, the spherospheres in the core of the scaffold were surrounded by the matrix preserving their spheroidal phenotype with healthy cell nuclei and actin filaments (Figure 9D, E), confirming the successful seeding of enriched-CSCs by injection-based method.

The mechanical properties of the 3D CSCs niche model were also evaluated up to 14 days by DMA analysis, with the final aim to assess if there were changes in viscoelasticity, compared to the *blank* as scaffold as is (Figure 9F). The capability to maintain the 3D structure together with an adequate and stable elastic modulus during cell culturing are essential properties for cellular scaffolding²⁷⁷. In detail, Young's Modulus (E) is an intrinsic material parameter used to describe the relationship between the unitless percent elongation of a material, the strain (ϵ), and a force applied per area, that is the stress (σ), expressing the linear relationship $E = \sigma/\epsilon$ in units of Pascal²⁷⁸; stiffer materials exhibit higher Young's Modulus value compared to softer materials. Considering that the development of materials with mechanical properties close to that one of the real bones (i.e. the Young's Modulus of the calcified bone is on the order of ~ 5 GPa)²⁷⁸ is still a huge challenge and that the reproduction of a tumoral bone tissue is even more arduous due to its extremely high histological heterogeneity which involves the simultaneous presence of soft non mineralized zones (osteoid matrix) next to stiffer areas⁴, the Young's Modulus of the 3D CSCs niche model has been investigated to evaluate if there were any changes in mechanical behavior of the MgHA/Coll scaffold during cell culturing due to the presence of cell media, cellular adhesion and metabolism²⁷⁷.

The results did not show significant differences in both groups, close and open injection, up to 14 days of culture, compared to the *blank* (Figure 9F), assessing the mechanical stability of the 3D CSCs niche model.

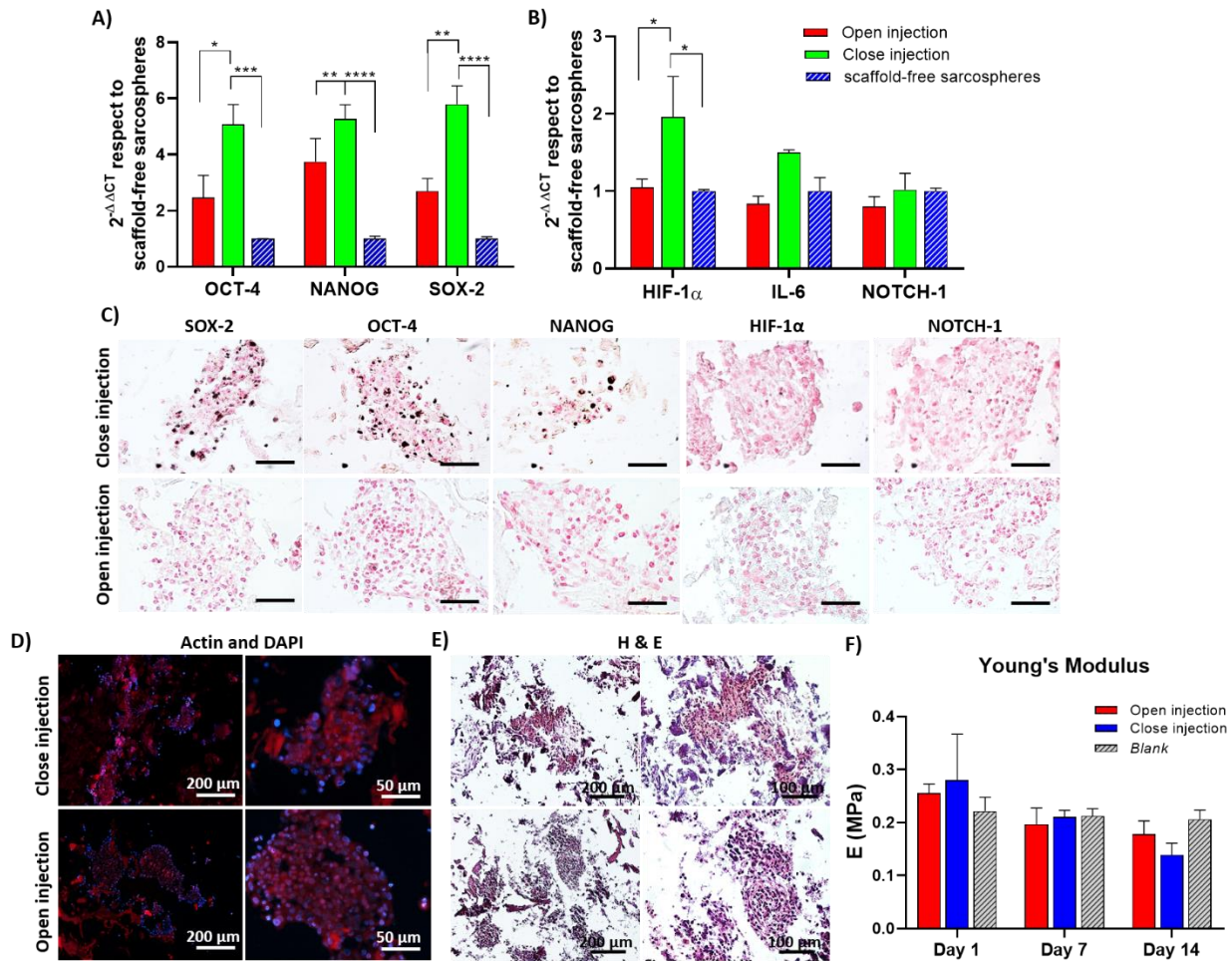


Figure 9. The *in vitro* 3D CSCs niche model²²⁸. qRT-PCR of stemness (A) and CSCs niche-related markers (B) as fold-change expression relative to scaffold-free G1. The graphs show the mean $\pm$ SEM (N=3); (C) immunohistochemistry analysis of stem and CSCs niche-related markers. Positivity in brown, cell nuclei in pink. Scale bars 50 μ m (N=3); (D) Actin and DAPI staining with F-actin in red and cell nuclei in blue (N=1); (E) H&E staining with cytoplasm in pink and cell nuclei in violet (N=1); (F) Mechanical properties of the 3D CSCs niche model by DMA analysis reporting the Young's Modulus (E) up to 14 days. *p value $\leq$ 0.05, ** p value $\leq$ 0.01, *** p value $\leq$ 0.001, **** p value $\leq$ 0.0001.

4.2.3. The evaluation of *in vivo* 3D CSCs niche model in CAM model, proof of concept

As last optimization step, the potential tumorigenic properties of the 3D CSCs niche model, open and close injection, were assessed on the Chorioallantoic Membranes (CAMs) models of chick embryo, a highly vascularized *in vivo* system that enables the effective formation of solid tumours in a short time. In this study²²⁸, a proof of concept of the capacity of inducing the tumour development *in vivo* by the 3D CSCs niche model has been investigated. The models were developed *in vitro* and culture

for 7 days before being engrafted on a small vein of the membrane of the chicken embryos at EDD6 (see paragraph 3.4.3.). The viability of the embryos was monitored until EDD14, where both, close and open injection, showed a 50% survival rate compared to the total amount of engrafted models (100%). The results overall demonstrated the successful tumorigenic properties of both groups of 3D CSCs niche model after 7 days of *in vivo* growth (Figure 10 and 11). The 3D CSCs niche model reached a greater volume compared to the CTR, namely the sarcospheres-free scaffold, indicating the development of a tumour-like mass around the engrafted models (Figure10A). This was confirmed by the increased weight of both, open and close injection groups, approximately 3.4 and 2.2-fold at EDD14 compared to their starting weight (Figure 10B).

The histological analysis by Alizarin Red and Von Kossa showed the development of an extensive mineralization in the close injection, which is homogeneously spread close to the membrane of the capsule, compared to the open injection and the CTR (Figure 10C). Moreover, the morphological analyses showed clearly visible sarcospheres with the preservation of the spheroidal phenotype in the open injection, compared to the close injection, wherein the cells seem to be more embedded/internalized in the system (Figure 10C, D).

These results, namely the tumour growth, the preservation of the spheroidal phenotype and the increased mineralization, overall represented significant marks of the tumour development potential²⁷⁹.

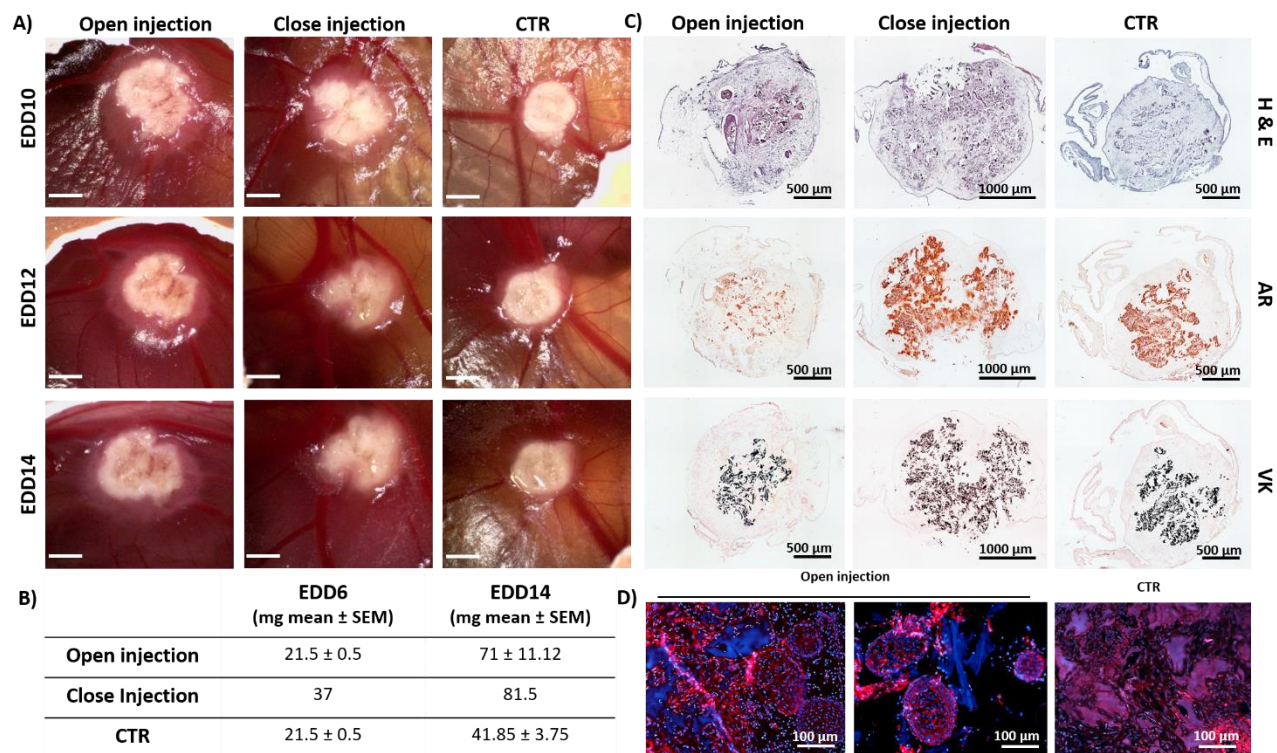


Figure 10. Growth, weight and histological analysis of tumour grafts²²⁸. (A) Growth observation of 3D CSCs niche grafted model during the experimental window. Scale bar: 2 mm. (B) Table of the weight mean in mg $\pm$ SEM; no SEM is reported for close injection as one replicate was analyzed. (C) H&E (pink cytoplasm, violet cell nuclei), Alizarin Red (red calcium deposits) and Von Kossa (black calcium salts, pink cell nuclei) histological staining in the harvested 3D CSCs niche models. (D) Actin and DAPI staining of the harvested 3D OS models; cell nuclei in blue, F actin filaments in red.

The immunohistochemical analysis was performed on the harvested samples, reporting clear positivity for OCT-4, SOX-2 and CD133²⁸⁰ stem markers in both groups of 3D CSCs niche model, compared to the CTR, assessing the preservation of CSCs features *in vivo* and the development of a stem-like tumour (Figure 11). Positivity for Ki67, as cell proliferation regulator and marker for tumour diagnosis²⁸¹, and PECAM-1 in both groups of 3D CSCs niche model together with a slight positivity for vWF in the open injection were also observed, compared to the CTR (Figure 11), confirming the development of the tumour. Indeed, tumor neovascularization involves the formation of new blood vessels, which are crucial for tumour progression²⁸²; in this context, PECAM-1, the gene encoding for CD31 transmembrane glycoprotein, represents an early marker for tumour-induced angiogenesis, indicating processes like angiogenesis and vascular permeability²⁸³. Therefore, PECAM-1 positivity in both groups of 3D CSCs niche model confirms the early-stage angiogenesis compared to CTR, while the gene upregulation of HIF-1 α and IL-6 demonstrated their role in contributing to CSCs-mediated production of angiogenic factors and vasculogenic mimicry²⁸². Conversely, vWF, typically expressed in the advanced tumour stage, was observed minimally in the open injection, confirming the development of an immature neovascularization^{284, 285}.

In conclusion, in this study²²⁸ we successfully designed, developed and characterized a more predictive *in vitro* 3D CSCs niche model with *in vivo* tumorigenic properties, which can lead to important advancements in oncological research and open brilliant perspective for future implementation of more complex systems (*i.e.* co-culture, matrix-functionalization) able to mimic specific malignant behaviours (metastatic process, drug resistance, etc.), increasing the knowledge of tumour biology and pushing the discovery of new therapeutic strategies for clinical applications.

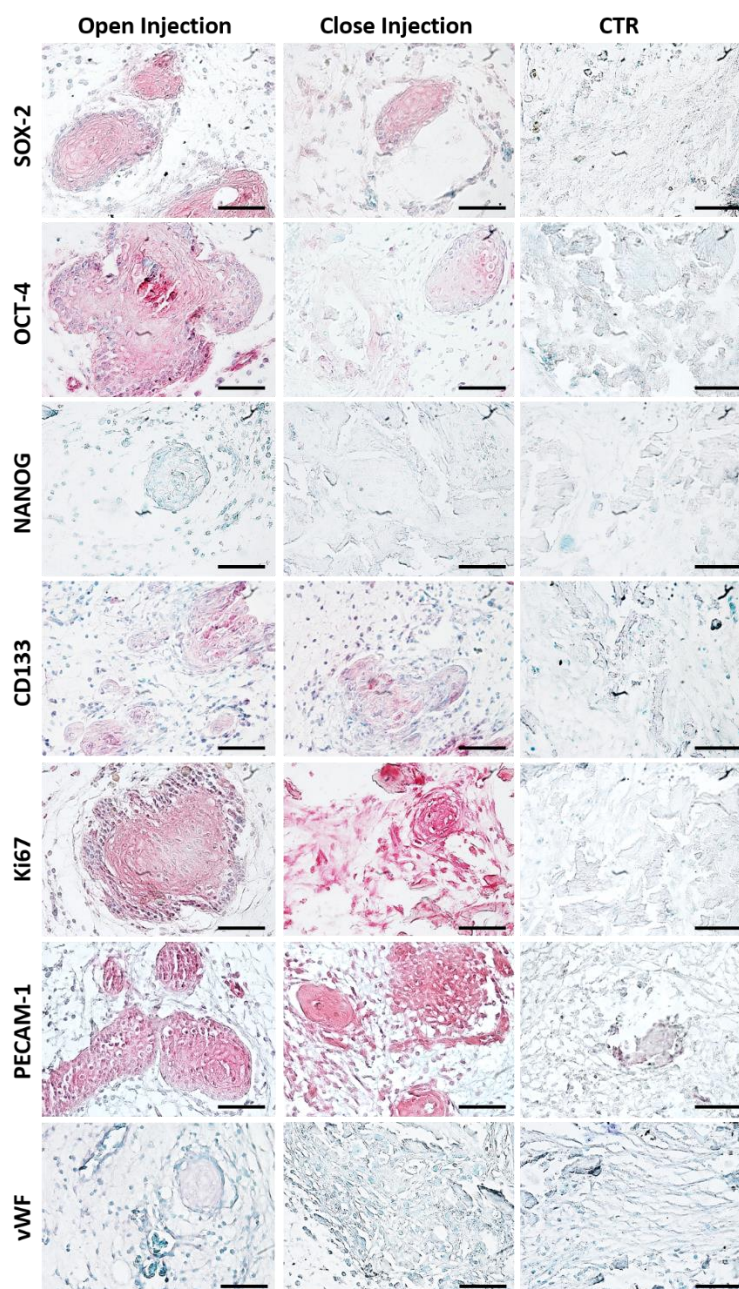


Figure 11. Immunohistochemistry analysis of the harvested 3D CSCs niche model²²⁸. Immunolocalization of SOX-2, OCT-4, NANOG, CD133, Ki67, PECAM-1 and vWF in the harvested 3D CSCs niche model. Positivity in pink, cell nuclei in light blue. Scale bars 50 μ m.

4.3.The Drug Resistance of the 3D CSCs niche model

Following the successful optimization and development of the 3D CSCs niche model, the further advancement of the study focused on evaluating the drug resistance phenomenon under both *in vitro* and *in vivo* CAM model conditions. Specifically, the close injection approach has been used for this study and developed after size optimization as reported in paragraph 3.4.3.1. The model was tested with cisplatin and doxorubicin, as common chemotherapeutics typically used in the clinic, and with dibenzyl diselenide (DBDS), as a promising organic Se candidate for its anti-neoplastic activity kindly provided by Prof. Valerio Voliani of the Center for Nanotechnology Innovation, Istituto

Italiano di Tecnologia (IIT), in Pisa, Italy¹¹³. For the *in vivo* study, a concentration of 830, 268 and 223 μM of cisplatin, doxorubicin and DBDS, respectively, resulting from the human-animal conversion formula²⁴² were used (see paragraph 3.5.1.). For the *in vitro* study, the concentration of 83, 27 and 23 μM for cisplatin, doxorubicin and DBDS, respectively, derived from a division by 10 of the *in vivo* concentrations, were used. The drug-free condition was used as a control group (CTR) (see paragraph 3.5.1.).

4.3.1. The evaluation of *in vitro* Drug Resistance of the 3D CSCs niche model

For the *in vitro* study, the 3D CSCs niche model was developed, and the drugs were added at the concentrations of 83, 27 and 23 μM for cisplatin, doxorubicin and DBDS, respectively, as reported in paragraph 3.5.1. After 7 days of growth *in vitro*, compared to the drug-free condition (CTR), the models were harvested after 24 and 96 hours of incubation for the evaluation of cell viability, gene and protein expression, and cell morphology.

The cell viability of the 3D CSCs niche model was investigated by MTT assay (Figure 12). The results showed that the models suggested a higher tolerance only towards cisplatin up to 24 hours of treatment, reporting no significant differences compared to CTR and significantly higher cell viability compared to Doxorubicin and DBDS (p value ≤ 0.0001). The same trend was preserved at 96 hours, where cisplatin showed significantly lower cytotoxicity compared to Doxorubicin and DBDS (p value ≤ 0.0001), however a significant reduction of cell viability was showed compared to CTR (p value ≤ 0.0001). The behaviour was different for the models treated with Doxorubicin and DBDS, where a significant decrease of cell viability was reported starting from 24 hours of treatment up to 96 hours, compared to CTR (p value ≤ 0.0001) (Figure 12).

These preliminary results suggest the potentially higher resistance to cisplatin by the 3D CSCs niche models, compared to other treatments; however, the study is still a work in progress and more specific investigations on drug resistant and cell cycle-related markers will be considered for the analysis.

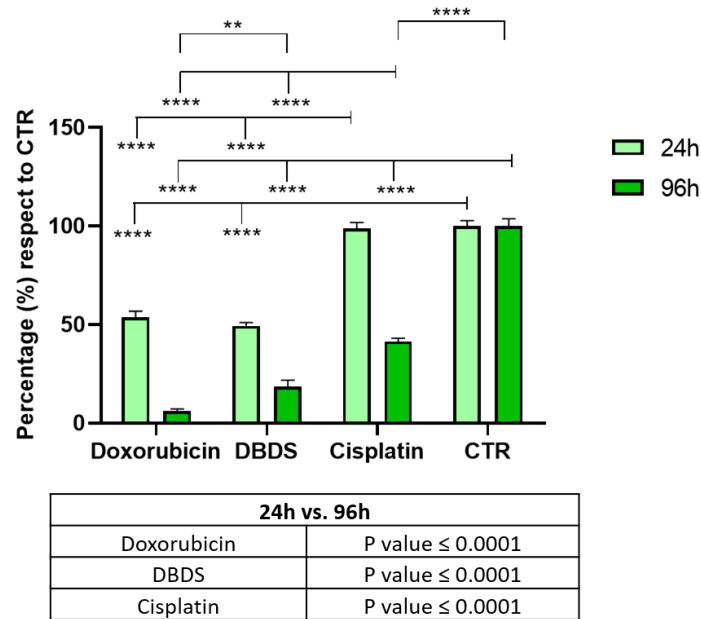


Figure 12. Cell viability by MTT assay. Cell viability of the 3D CSCs niche model *in vitro* after 24 and 96 hours of treatment. The graph shows the mean $\pm$ SEM. Significant differences among treatments at each time point are reported in the graph. Significant differences among time points for each treatment are reported in the table. **p value ≤ 0.01 and ***p value ≤ 0.0001 .

4.3.2. The evaluation of *in vivo* Drug Resistance of 3D CSCs niche model in CAM models

The 3D CSCs niche model has been grafted in CAM models to investigate whether the tumour developed *in vivo* was suitable to resemble the drug resistance phenomenon of OS.

Before proceeding with the experiment, the protocol used to obtain the 3D CSCs niche model for the *in vivo* study (as reported in paragraph 3.4.3.1.) was further optimized *in vitro* in terms of size reduction (Figure 13) to improve the repeatability of the 3D seeding procedure and the post-grafting yield of survival rate. Starting from the original MgHA/Coll scaffold ($\varnothing$: 9mm; h:6mm) (Figure 13A), final models with $\sim \varnothing$:3 mm; h: 3 mm; 1.5 mm thick size, were successfully obtained by exploiting a protocol which consisted of 3 transversal sectionings and 1 pinching (3 mm puncher) (Figure 13B). The cell viability of the sarcospheres after injection-based seeding in the optimized models, developed as reported in paragraph 3.4.3.1, was preliminarily evaluated after 7 days of culture, showing overall good cell viability and the successful injection-based seeding of the sarcospheres on the scaffolds (Figure 13C). Most importantly, the weight of the models was assessed after 7 days of culture, confirming the successful development of 3D CSCs niche models with overall ≤ 30 mg weight, being suitable for the *in vivo* grafting in CAM models (Figure 13D).

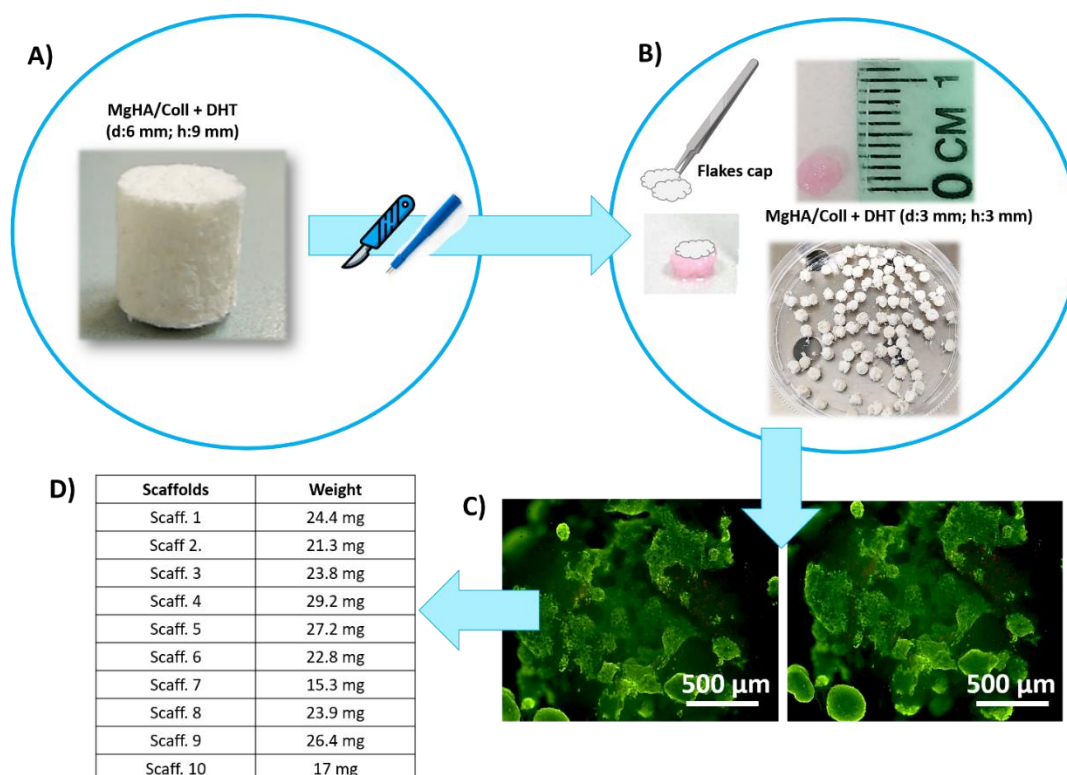


Figure 13. Schematic representation of the optimized protocol used to obtain the 3D CSCs niche model for the grafting *in vivo* CAM models. (A) Starting MgHA/Coll scaffold's size; (B) Final MgHA/Coll scaffold's size and development of the injection-based approach for 3D CSCs niche model; (C) Preliminary cell viability evaluation of the optimized 3D CSC niche model by Live and Dead Assay. Live cells in green, dead cells in red; (D) Weights (mg) of the optimized 3D CSCs niche models after 7 days of culture.

For the drug resistance study, the fully optimized 3D CSCs niche model was cultured for 7 days before the engrafting in CAM models at EDD6 (see paragraph 3.4.3.1.). The eggs were incubated until EDD10 to make the tumour develop, followed by the addition of treatments by dripping the drug solutions on the tumour mass at the concentration of 830, 268 and 233 µM for cisplatin, doxorubicin and DBDS, respectively as reported in paragraph 3.5.1. The 3D CSCs niche model was compared to the drug-free samples and sarcospheres-free scaffold engrafted as *blank* to assess the tumour's successful development. The models were monitored at EDD6, EDD10, EDD12 and EDD14, up to 96 hours of treatment, before harvesting, weighting and treatment for different biological analyses. The tumour monitoring allowed us to observe the successful engrafting of 3D CSCs niche model in CAM model without observing any sign of inflammation, necrosis or death of the chick embryos throughout the experiment, according to the results of the *in vivo* proof of concept (Figure 14). As the first analysis, the development of a fully vascularized tumour-like mass in the chick embryo incorporating the 3D CSCs niche model was observed and assessed starting from EDD6, compared to the *blank* (Figure 14A). Indeed, a significantly different tumour weight has been shown by 3D CSCs niche model, compared to the *blank* at EDD14 (p value ≤ 0.05) (Figure 14B) and this difference

in terms of tumour growth was also confirmed by the volumetric analysis (Figure 14C), showing a continuous increase of tumour volume in the 3D CSCs niche model (i.e. CTR as a drug-free condition) from EDD6 to EDD14 ($p \text{ value} \leq 0.01$), with a significant difference at EDD14 ($p \text{ value} \leq 0.5$) compared to the *blank*, whose volume remains stable. These results overall corroborate the successful engrafting of the 3D CSCs niche model and its tumorigenic properties *in vivo*, confirming the results of the proof of concept.

Regarding the different treatments, the results showed that the drug administration overall did not significantly reduce the volume of the tumour from the day of the treatment (EDD10) up to EDD14 (Figure 14C, C1, D). In detail, a significant increase in tumour volume has been observed in the presence of Doxorubicin and DBDS at EDD12 and EDD14, with respect to EDD6 ($p \text{ value} \leq 0.01$, $p \text{ value} \leq 0.001$ for Doxorubicin and $p \text{ value} \leq 0.05$ for DBDS) (Figure 14C1), with significant differences for Doxorubicin compared to the *blank* at EDD12 and EDD14 ($p \text{ value} \leq 0.05$) (Figure 14C), suggesting the overall total absence of any post-treatment effect on the tumour's growth. The exception is for cisplatin, which after the significant increase in tumor volume by EDD10 compared to both EDD6 ($p \text{ value} \leq 0.05$) and the CTR ($p \text{ value} \leq 0.05$), indicating tumor growth before treatment, led to a not significant reduction in volume by EDD14 (Figure 14C1).

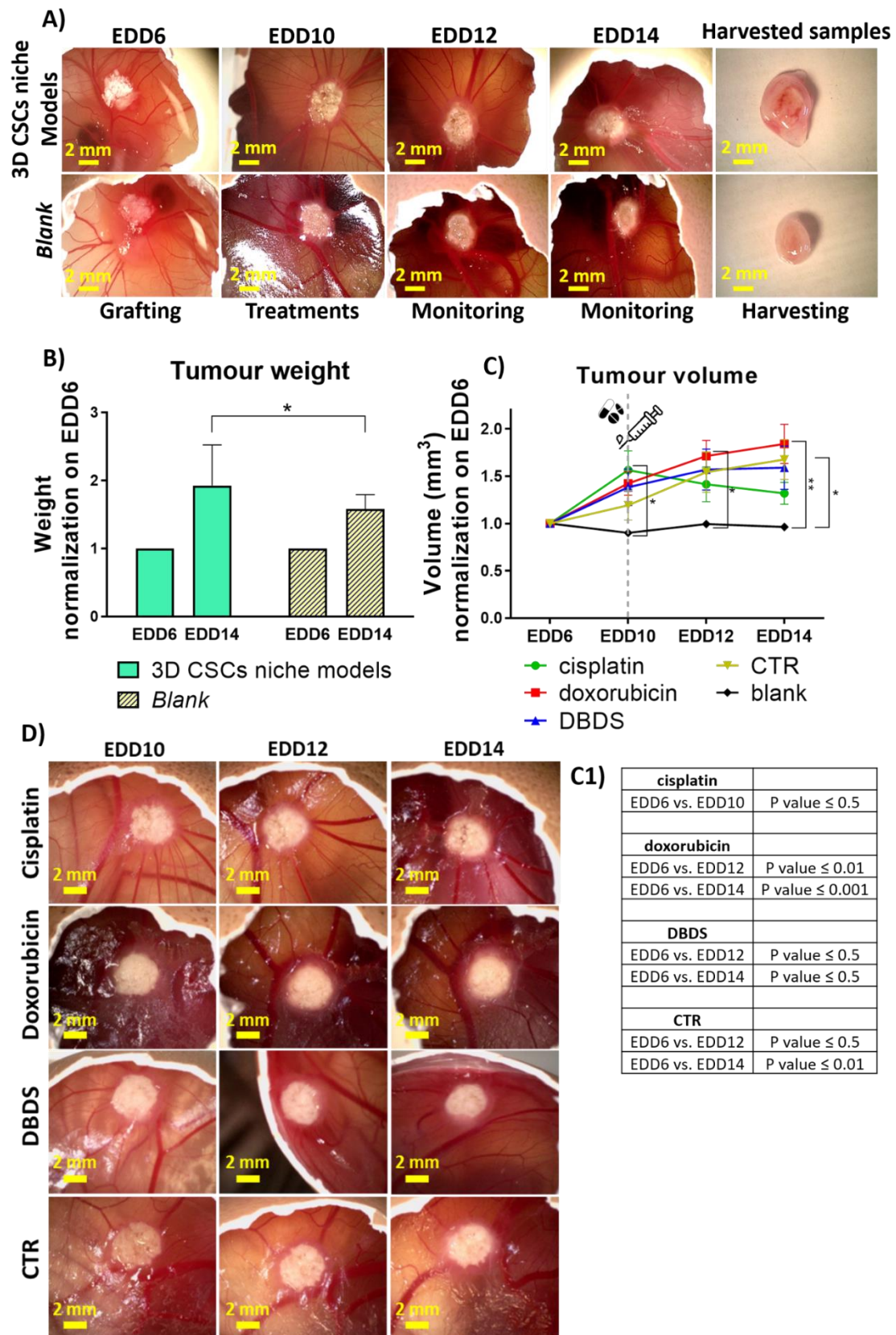


Figure 14. Tumour monitoring of *in vivo* drug resistance evaluation. (A) Representative panel of tumour growth and size of 3D CSCs niche model at EDD14, compared to the *blank*. (B) Analysis of the weights of the tumour masses of 3D CSCs niche model and the *blank* at EDD14. Data are reported in the graph as a mean of the weight (mg) normalized on pre-grafting EDD6 $\pm$ SEM. *P value ≤ 0.05 ; (C) Analysis of the volume trend of the tumour mass of 3D CSCs niche

model from EDD6 to EDD14. Data are reported in the graph as volume mean (mm^3) normalized on EDD6 $\pm$ SEM. Statistically significant differences over time are reported in the table C1. *P value ≤ 0.05 , **p value ≤ 0.01 . (D) Tumor monitoring of the engrafted 3D CSCs niche model treated with drug at EDD10, EDD12 and EDD14, compared to CTR.

The Alizarin Red and Von Kossa stainings showed an increasing trend in mineralization in the drug-treated samples, compared to CTR (Figure 15A, images B, G, N, S and C, H, O, T), with a significant difference in DBDS reported by Von Kossa staining, compared to CTR (p value ≤ 0.05) as confirmed by the semi-quantitative analysis of images (Figure 15B). These results agree with the long-recognized phenomenon of an increase in the tumour's mineralization during chemotherapy in osteosarcoma patients²⁸⁶. Indeed, some investigators hypothesized that the increased mineralization indicates a reduced response to chemotherapy²⁸⁶⁻²⁸⁸, suggesting the potential trigger of drug resistance phenomenon in the *in vivo* models treated with drugs.

The Alcian Blue and Safranin-O stainings were performed to investigate the nature of the tumour-like mass surrounding the engrafted models, however, no significant differences were observed among samples and the absence of polysaccharides and proteoglycans was assessed (Figure 15A).

The gene expression of stem and environmental markers of CSCs was also analysed with the final aim of assessing the presence of any changes in their phenotype and niche after treatments (Figure 15C). The results reported an upregulation trend of OCT-4, SOX-2 and HIF-1 α , together with significant overexpression of NANOG only in the cisplatin, compared to CTR (p value ≤ 0.01) and other treatments (p value ≤ 0.05 and ≤ 0.01); this behaviour can be related to the triggering of drug-resistant mechanism in the treated model (Figure 15C), such as the stimulation of stem properties and hypoxic conditions. Indeed, beyond being responsible for the cellular adaption to hypoxia, HIF-1 α 's transcriptional targets may improve the stemness properties of CSCs²⁷⁵ and induce the drug resistance phenomenon by affecting drug transport (e.g. ATP-binding cassette ABC transporters) or the response to the drug (e.g. apoptosis, autophagy)¹²⁰. However, no significant differences were reported for DBDS and Doxorubicin. The gene expression of more specific drug-resistant, cell cycle and cell death mechanism regulators, is still working in progress and will be considered for the study in order to elucidate the effect of each drug on the tumour mass.

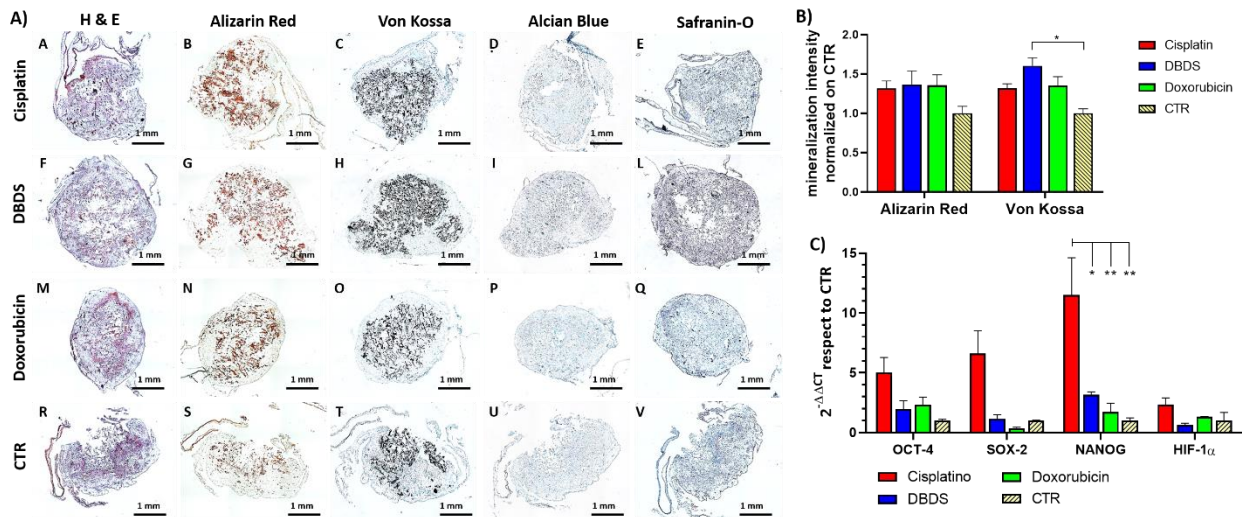


Figure 15. Histological analysis and gene expression of *in vivo* drug resistance. (A) Histological evaluation of harvested CSCs niche model by H & E (A, F, M, R), Alizarin Red (B, G, N, S), Von Kossa (C, H, O, T), Alcian Blue (D, I, P, U) and Safranin-O (E, L, Q, V); (B) semi-quantitative analysis of mineralization by Alizarin Red and Von Kossa staining by ImageJ software. The graph shows the mean $\pm$ SEM; (C) gene expression as fold-change expression relative to CTR. The graphs show the mean $\pm$ SEM. *P value $\leq$ 0.05, **p value $\leq$ 0.01.

The immunolocalization of different tumor markers was performed by immunofluorescence analysis (Figure 16A) and evaluated in terms of fluorescence intensity (Figure 16B).

The collagen type I was investigated for its role in the OS, as it has been demonstrated that the collagen enrichment in OS tissues could mediate the tumour development, leading to increased malignancy. Most importantly, the collagen can influence the chemotherapy efficacy through specific receptors suggesting that tumour-associated collagen might influence tumour progression and chemotherapy efficacy in the TME²⁸⁹. Our results clearly showed positivity for collagen type I in all the conditions tested (Figure 16A) with the presence of an increasing trend of the fluorescence intensity in the treated models, compared to CTR (Figure 16B), in line with the literature reporting the association of collagen to the potential development of the drug resistance phenomenon.

The proliferation regulator Ki67, the transmembrane glycoprotein CD44 and the pluripotency marker OCT-4 reported a slight positivity in all the conditions tested, without any significant differences, suggesting high proliferation rates and stem properties in the developed tumours (Figure 16A and B). Indeed, CD44 is a well-recognized marker linked to the self-renewal capacity of CSCs and the maintenance of CSC populations, assuming a relevant role in tumour initiation, progression and chemotherapy resistance^{290, 291}; again, the higher expression of tumour proliferation Ki67 is linked to increased proliferation rates²⁹². Our results pointed out that no changes were observed in terms of stemness properties and proliferation in the treated models, compared to CTR, indicating that the treatment with drugs do not compromise the tumor properties. Interestingly, HIF-1α was highly

expressed in the samples treated with cisplatin and DBDS, compared to CTR (Figure 16A and B), with a significant difference in cisplatin (p value ≤ 0.05) (Figure 16B).

A similar trend was reported for stem marker CD133²⁸⁰ (Figure 16A), whose expression was significantly upregulated in the samples treated with Doxorubicin and DBDS, compared to CTR (p value ≤ 0.001 and ≤ 0.0001) (Figure 16B). An upregulation trend was also observed in the cisplatin-treated sample, even if it was significantly lower compared to Doxorubicin (p value ≤ 0.0001) and DBDS (p value ≤ 0.05) (Figure 16B). Indeed, CD133, also known as prominin-1, is a glycoprotein which is used in combination with other markers to identify CSCs in tumours^{293, 294} and CD133-expressing CSCs have been associated with higher self-renewal potential and poor prognosis and survival rate²⁹⁵; most importantly, high expression of CD133 is associated with drug resistance as its overexpression had been found associated to increased expression of ABC transporter ABCG2²⁹⁶. The observed gene expression profile can be considered a molecular behaviour induced by the initial triggering of the drug resistance mechanism.

The *in vivo* drug-resistant study confirmed the tumorigenic properties of our models and suggested the triggering of drug-resistant mechanisms in the treated samples, compared to CTR. Considering this, the data must be confirmed by more quantitative analysis.

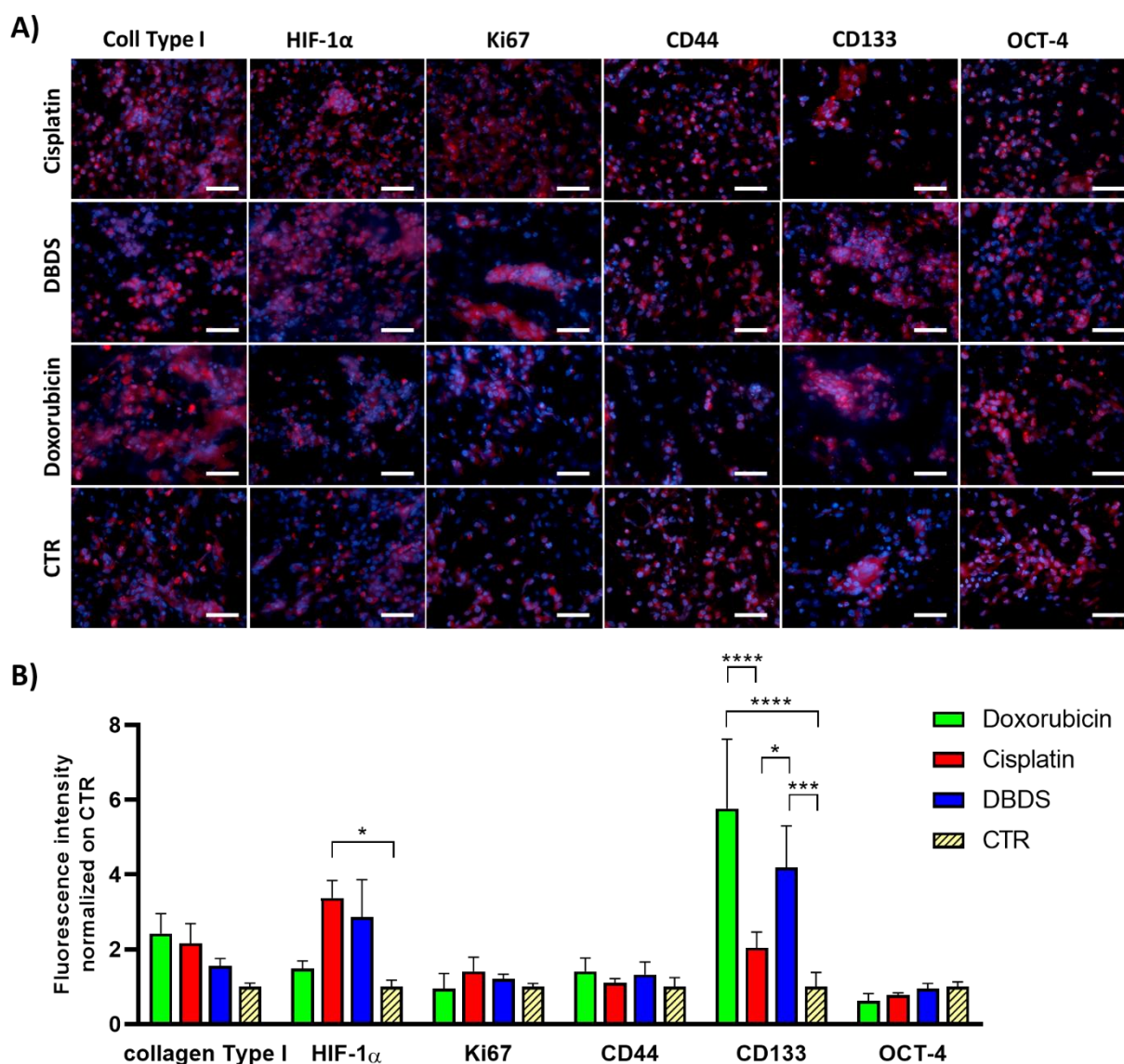


Figure 16. Immunofluorescence analysis of *in vivo* drug resistance. (A) Immunofluorescence analysis in the treated samples compared to CTR. Scale bar 30 μ m; (B) semi-quantitative analysis of the fluorescence intensity of the immunofluorescence by ImageJ software. The graph shows the mean $\pm$ SEM. *P value $\leq$ 0.05, ***p value $\leq$ 0.001 and ****p value $\leq$ 0.0001.

4.4. The *in vitro* 3D co-culture model of OS

Osteosarcoma's inherent tumor heterogeneity presents a significant challenge to understanding its pathology and developing effective treatments. A dynamic extracellular matrix (ECM), together with a network of blood vessels, diverse cell types (e.g. MSCs), and varying environmental conditions, plays a key role in osteosarcoma progression and contributes to drug resistance¹. The development of more predictive *in vitro* models that capture this complexity is essential for obtaining reliable data. To address these challenges, this study focused on developing a 3D co-culture system of osteosarcoma, incorporating multiple cell types and scaffold-based structures that better replicate the

heterogeneity of the Tumour Microenvironment (TME). Specifically, iBM-MSCs were used as one cellular components having a key role in the OS origin as well as in the tumour progression through their potential differentiation into Tumoral-MSCs (T-MSCs) or Cancer Associated Fibroblasts (CAFs). The specific interaction between iBM-MSCs and GFP-MNNG HOS osteosarcoma cells and cell-ECM was considered in this study developing a 3D co-culture model combining the MgHA/Coll scaffold as bone-like ECM and the two cell types at 1:1 ratio. The model was deeply investigated at 7 and 14 days of culture, compared to the 3D mono-culture conditions and 2D scaffold-free conditions.

The results successfully showed a good interaction between tumour cells and iBM-MSCs, with both cell types reporting healthy cell nuclei and cytoskeleton, together with huge scaffold colonization in all 3D scaffold-based conditions starting from 7 days of culture (Figure 17).

The direct interaction between the tumour cell and the iBM-MSC was observed by the Actin and DAPI staining at both time points (Figure 17A) confirming that both the cell types were able to grow, interact and colonize the scaffold. This is also demonstrated by the SEM analysis, where the scaffolds appeared completely covered by a dense cellular network (Figure 17E).

The cellular ability in colonizing the scaffold was also quantitatively analysed to investigate if the cellular migration and invasion into the matrix were affected in co-culture conditions compared to mono-cultures. Briefly, after DAPI staining each 3D OS model was sectioned by scalpel and the migrating cells from the seeding surface to the core of the matrix were observed at the fluorescent microscope by cell nuclei identification as reported in the representative images (Figure 17B and D). The distance travelled by the cells has been calculated in μm and reported in the graph (Figure 17C). The results showed that the tumour cells had a significantly higher migrative potential compared to iBM-MSC alone ($p \text{ value} \leq 0.001$), according to the well-recognized metastatic potential of osteosarcoma cells²⁹⁷. Interestingly, the co-culture condition showed significantly higher cell colonization compared to iBM-MSC alone ($p \text{ value} \leq 0.05$). One of the possible explanations for this behaviour is that a change in MSCs behaviour when cultured in the presence of tumour cells occurred^{298, 299}, which in turn could be ascribable to the MSCs well-recognized education into tumour-associated MSCs (T-MSCs) and CAFs acquiring higher migrative and invasive potential^{47, 300}. On the other side, the cellular colonization of co-culture conditions is significantly lower compared to tumour cells alone ($p \text{ value} \leq 0.05$), suggesting the potential role of MSCs in inhibiting tumour invasiveness and metastasis^{301, 302}. Many controversies are reported in the literature on the role of MSCs in cancer

and, a more detailed analysis should be performed to clarify the effect of this interaction.

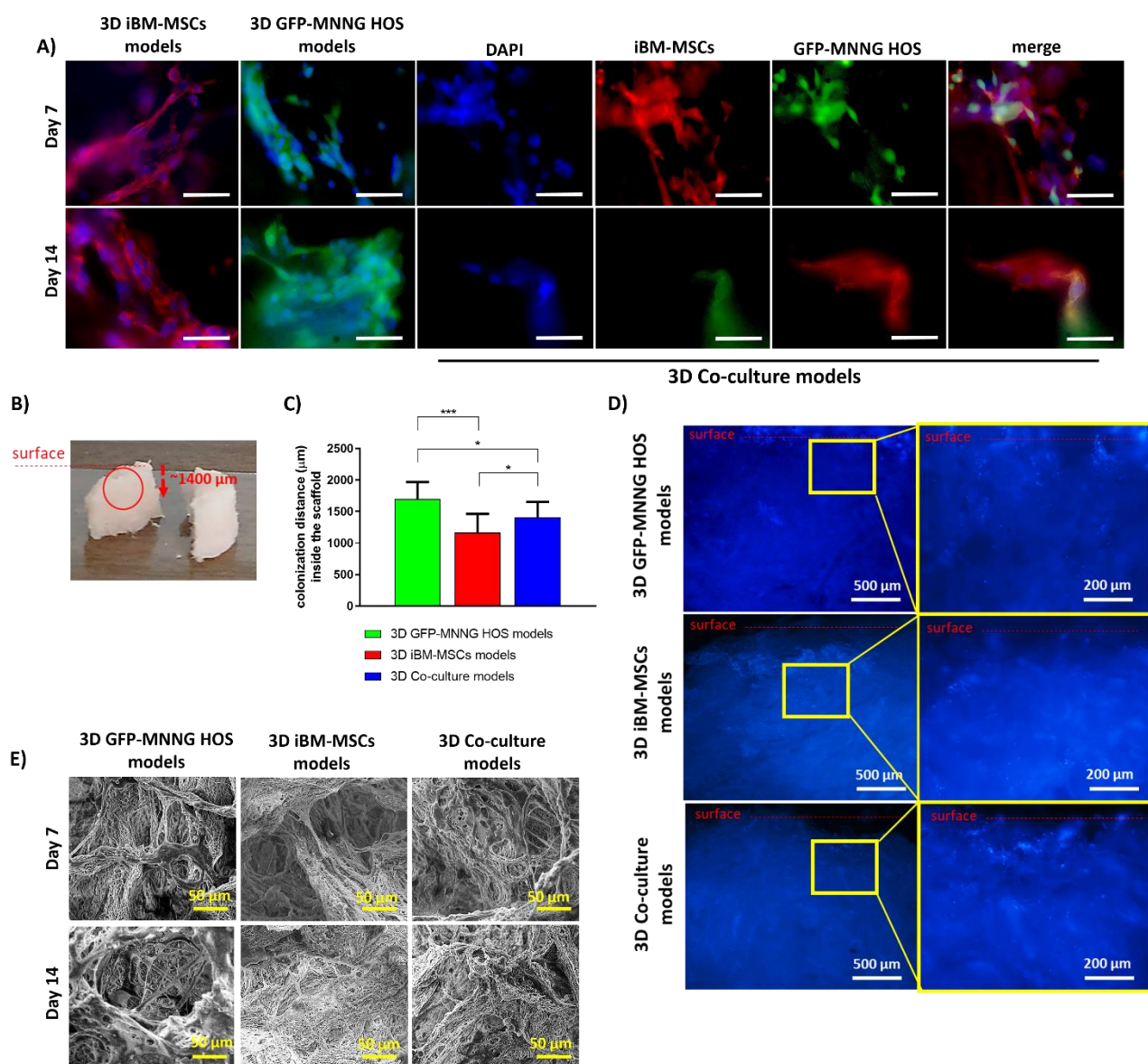


Figure 17. Cell morphology and colonization of the *in vitro* 3D Co-culture models of OS. (A) Actin and DAPI staining of the 3D co-culture models and 3D mono-culture conditions at 7 and 14 days of culture. GFP-MNNG HOS in green, iBM-MSCs in red and cell nuclei in blue. ActinRed 555 ReadyProbes Reagent was used to stain F-actin filaments and distinguish iBM-MSCs. Scale bars 30 μm ; (B) Representative image of the sectioning and analysis of the 3D models for the evaluation of the scaffold colonization by cells; (C) Colonization distance travelled by the cells from the seeding surface to the core of the scaffold at day 7 of culture. The data are graphically plotted as mean $\pm$ SEM. *p value ≤ 0.05 , *** p value ≤ 0.001 ; (D) Representative panel of scaffold colonization by the cells at day 7 of culture. On the left a low magnification of the inner side, and on the right a higher magnification of a specific area. The seeding surface is pointed by the red dotted line; (E) SEM analysis of the 3D co-culture models and 3D mono-culture conditions at 7 and 14 days of culture.

The gene expression profile has been investigated in this study to try to uncover the nature of the interaction between the two cell types (Figure 18 and 19).

Firstly, the expression of specific MSCs-surface markers was analysed to investigate the potential change in the mesenchymal phenotype under the different cell culture conditions (Figure 18). The results of the gene expression reported that in the co-culture conditions, all the MSC-surface markers (endoglin, CD90 and CD73) were significantly inhibited, compared to iBM-MSC alone, under both 2D and 3D scaffold-based conditions at both time points (Figure 18 A, A1, A2 and A3).

In detail, a significant downregulation of endoglin (p value ≤ 0.05 and p value ≤ 0.01), as well as CD90 (p value ≤ 0.001) and CD73 (p value ≤ 0.01 and p value ≤ 0.001) was reported in the co-culture at both time points under 3D scaffold-based conditions (Figure 18A, A2). The downregulated trend is reported also under 2D condition even if without statistically significant differences (Figure 18A1, A3). These results can indicate an alteration of the mesenchymal phenotype (Figure 18A, A1, A2 and A3); indeed, based on the minimal criteria established by the International Society for Cellular Therapy (ISCT), human MSCs are identified by the combined expression of endoglin, CD73 and CD90 cell-surfaces markers which must be expressed by $\geq 95\%$ of the cell population³⁰³. Although endoglin, or CD105, is a type I membrane glycoprotein whose expression has been shown to vary depending upon MSC source and culture time *in vitro*, it is established that its absence identifies differentiated MSCs with increased osteogenic gene expression^{304, 305}. The CD73 is an ecto-5'-nucleotidase that converts extracellular adenosine monophosphate to adenosine and it is expressed by MSCs in a different form compared to that one expressed by other cell types (i.e. endothelial cells, epithelial cells), making it a unique and specific marker for MSCs identification³⁰⁶. The CD90, also known as Thy1, is a glycosylphosphatidylinositol-linked protein involved in cell-cell and cell-matrix interactions that is highly expressed in all MSCs, irrespective of the source, and its expression has been related to the undifferentiated status of MSCs³⁰⁷.

The downregulation of these three markers indicates that a decrease of mesenchymal phenotype occurs in the presence of tumour cells and more detailed analyses are needed to uncover the fate of MSCs.

It's interesting to note that the expression of the pluripotency-related gene NANOG is significantly upregulated in the co-culture compared to iBM-MSCs alone in the 2D condition at both time points (p value ≤ 0.05) (Figure 18A1 and A3), while it was showed the same level in both co-culture and iBM-MSCs alone under 3D scaffold-based condition (Figure 18A and A2), indicating again that the presence of the matrix significantly affects gene expression. NANOG constitutes the core-regulatory network that suppresses differentiation-associated genes, thereby pointing out the maintenance of the pluripotency of MSC³⁰⁸; a study reported that the expression of NANOG can be upregulated as a result of a spontaneous fusion between a tumour cell and MSC, suggesting nuclear reprogramming and improving the tumour progression³⁰⁹. In our results, the stable trend of NANOG, or even

upregulated under 2D conditions, suggests the potential triggering of the tumoral phenotype of MSC. To investigate the effects on iBM-MSCs co-cultured with tumor cells, the expression levels of osteogenic markers RUNX-2, BMP-2, and ALP were analyzed (Figure 18 B, B1, B2 and B3). Our results reported the absence of MSCs osteogenic induction in the co-culture, compared to iBM-MSCs alone, grown under both 2D (Figure 18 B1 and B3) and 3D scaffold-based conditions (Figure 18B and B2) for 14 days, suggesting overall that the downregulation of cell-surface markers of iBM-MSCs is not related to their potential osteogenic differentiation. Despite the well-known role of the MgHA/Coll scaffold in stimulating the osteogenic commitment of MSCs^{310, 311}, the presence of the bone-like scaffold does not simulate an osteogenic differentiation when MSCs grown in co-culture condition.

In detail, even if RUNX-2 was expressed in the co-culture at 7 days in 2D conditions, the significant downregulation of BMP-2 and ALP was reported (p value ≤ 0.05), compared to iBM-MSCs alone (Figure 18B1); the same trend was showed in the presence of the bone-like scaffold, where BMP-2 was significantly downregulated (p value ≤ 0.05) and ALP expression was even undetectable in the co-culture (Figure 18B). At 14 days of culture, while the gene expression profile remained almost unchanged under 2D conditions (Figure 18B3), the downregulation of all osteogenic markers was highlighted in the co-culture in the presence of the MgHA/Coll scaffold (p value ≤ 0.0001), compared to iBM-MSCs grown alone (Figure 18 B2). These results assessed that the loss of the mesenchymal phenotype in the co-culture did not belong to their osteogenic commitment despite the presence of the bone-like scaffold.

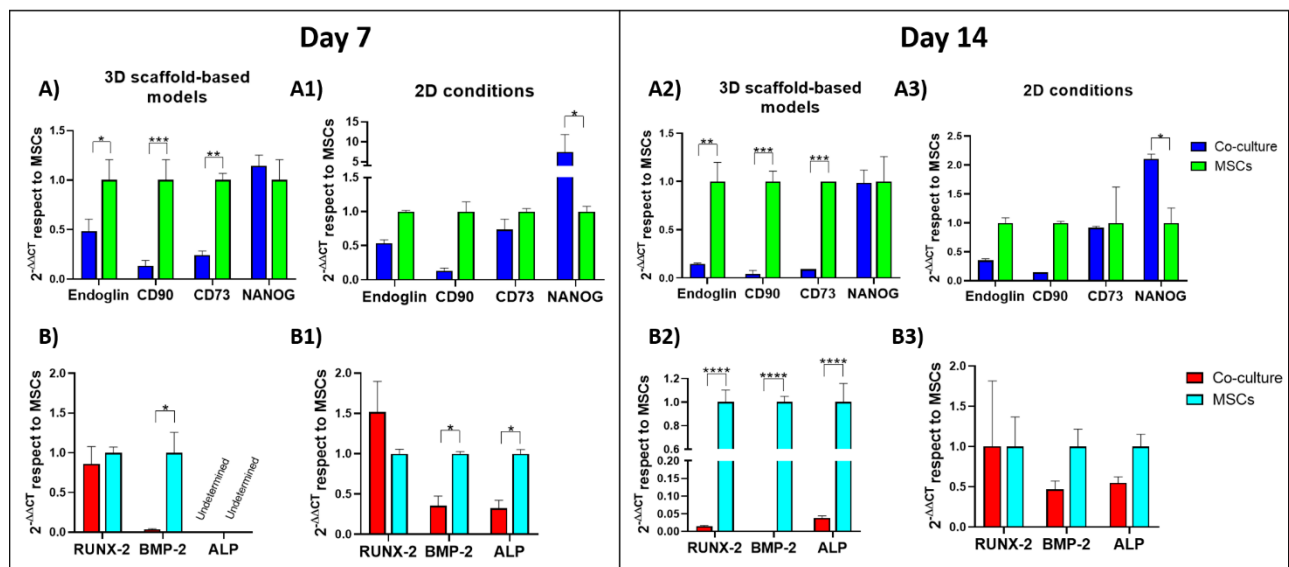


Figure 18. Gene expression of cell-surface MSC and osteogenic markers. Gene expression of the cell-surface markers of MSCs as fold-change expression relative to MSCs alone (A, A1, A2, A3); gene expression of the markers of the osteogenic pathway as fold-change expression relative to MSCs alone (B, B1, B2, B3). The graphs show the mean $\pm$ SEM. * P value ≤ 0.05 , ** p value ≤ 0.01 , *** p value ≤ 0.001 , **** p value ≤ 0.0001 .

The TME was also analysed for its significant role in affecting cellular behaviour in the OS, with the final aim to investigate if the co-culture condition was able to induce alterations of the environmental conditions and the activation of downstream signalling pathways, compared to mono-cultures. Several studies demonstrated that the three-dimensional cellular organizations have an intense impact on the morphogenesis, phenotype and gene expression profile of the cells³¹². Moreover, it is well known the role of the matrix and, its constituents and structure, in affecting the interaction of cells with the microenvironment and the triggering of individual cellular behaviours in terms of adhesion, differentiation, migration and invasion³¹³.

Considering this, the gene expression of some TME-associated markers was investigated (Figure 19). It is well-recognized that hypoxia plays a key role in the development and progression of the tumour⁴³; HIF-1 α , as a major transcription factor that mediates adaptive responses to low oxygen tension, is regulated by oxygen levels and it represents a hallmark of tumour hypoxia being widely expressed in solid tumour³¹⁴. The results reported no significant differences in the expression of HIF-1 α in all the cell cultures under 3D scaffold-based conditions, except for a significant downregulation in the co-culture at 14 days (p value ≤ 0.001), compared to 2D conditions (Figure 19). The observed reduction of HIF-1 α in the co-culture may reflect the inherent complexity of OS, where hypoxia heterogeneity in tumor cells is well-documented³¹⁴. Tumor hypoxia ranges from minimal to mild to severe, with distinct regulatory mechanisms operating at each level, highlighting the dynamic interplay between transient and chronic hypoxic cells^{43, 314}.

The expression of IL-6 and IL-8 showed a controversial trend. Briefly, at 7 days, the results reported that, under 3D scaffold-based conditions, both IL-6 (p value ≤ 0.001) and IL-8 (p value ≤ 0.0001) were significantly upregulated in iBM-MSCs alone (Figure 19A1), while no significant differences were observed in osteosarcoma cells (Figure 19A), compared to 2D conditions. IL-6 and IL-8 are pro-inflammatory cytokines which can be synthesized by various cell types, including monocytes, T-cells, fibroblasts, endothelial cells, MSCs and also tumour cells³¹⁵, affecting multiple biological activities. Their expression in tumour cells is recognized to generate a forward-feedback loop to stimulate cell proliferation, expansion, differentiation and drug resistance in the TME³¹⁶, on the other side IL-6 expression in MSCs is known to have a role in bone homeostasis and the maintenance of MSCs in an undifferentiated state³¹⁵. In line with the literature, our results confirm the expression of both cytokines in the osteosarcoma cells and iBM-MSCs; specifically, the cytokines upregulation in iBM-MSCs could depend on factors like their source and microenvironmental cues, emphasizing a context-dependent regulation of cytokines production³¹⁵ associated to the relevant role assumed by the MgHA/Coll scaffold.

However, it is possible to note that both cytokines are significantly downregulated in the co-culture (p value ≤ 0.0001 for IL-6; p value ≤ 0.001 for IL-8) under 3D scaffold-based conditions, compared to 2D ones (Figure 19A2). This trend is further highlighted at 14 days, where the significant downregulation of both cytokines was reported in all 3D scaffold-based cell cultures, compared to 2D conditions (p value ≤ 0.05 for osteosarcoma cells; p value ≤ 0.0001 for co-culture). These findings contrast with the evidence which reports that co-culturing MSCs with tumor cells typically results in elevated levels of the pro-inflammatory cytokine IL-6, promoting tumor growth and poor therapeutic outcomes⁵⁸. Additionally, OS cells are known to secrete IL-8, forming a signaling loop that induces IL-8 expression in MSCs, which in turn amplifies IL-8 production in OS cells⁴⁷. Nevertheless, it has been shown that the cytokines involved in the pro-cancer tumor microenvironment (TME) during tumor progression can exhibit temporal specificity, as different cells may release distinct pathogenic cytokines at specific stages of disease development, indicating time-dependent production^{315, 316}. In this context, the presence of the bone-like matrix and its components likely influence the timing of cytokine release compared to 2D conditions. This was evidenced by the expression level of IL-6 and IL-8 observed after 7 days of culture, which remarkably disappeared by 14 days in the presence of the scaffold. Such findings align with the widely recognized higher reliability of results obtained in 3D conditions, compared to the often overestimated outcomes of 2D models¹³⁵. On the other side, it is recognized that the MgHA/Coll is highly mimetic of the healthy bone tissue^{223, 240} and improvements in terms of pathological biochemical composition and structure are necessary to design a tumoral-like bone matrix. Since the cytokines production is extremely microenvironmental context-dependent³¹⁵, the absence of specific tumoural patterns in the matrix could be partly responsible for the absence of cytokines production at 14 days.

The gene expression of the Cancer-Associated Fibroblasts (CAFs) marker, α -SMA⁶⁵ (Alpha-Smooth Muscle Actin) was investigated to assess if the change in the mesenchymal phenotype was related to the potential differentiation of iBM-MSCs into CAFs after co-culturing with tumour cells. However, the results reported no significant differences between 3D and 2D conditions in both iBM-MSCs alone and co-culture at 7 days of culture (Figure 19A1 and A2), followed by a significant reduction of α -SMA in the co-culture in 3D scaffold-based models, compared to 2D conditions (p value ≤ 0.0001) (Figure 19B1 and B2). These results are in line with the downregulation of the cytokines observed as different studies reported the BM-MSCs differentiation into CAFs after co-culturing with osteosarcoma cell lines via increasing IL-6 expression, together with IL-8 levels in the TME, which further promotes the proliferation, migration and invasion of mesenchymal cells^{47, 58, 317}.

The gene expression results overall confirm that a change in the mesenchymal phenotype of iBM-MSCs occurred after being co-cultured with osteosarcoma cells and that this change seems to be not

related to their osteogenic commitment. The presence of the MgHA/Coll scaffold seems to introduce changes in the microenvironment, compared to 2D conditions, able to modify the cellular behaviours, confirming the significant role of the matrix. Improvements of the scaffold constituents are needed to mimic the tumoral matrix to regulate the release of cytokines and hypoxic conditions as well as the potential differentiation of MSCs into CAFs. RNAsequencing is in progress to investigate in detail the activation/deactivation of tumour pathways involved in the different cell cultures, with particular attention to cytokines production, thanks to the collaboration with professor Dominique Heymann of the Insitute of Cancerologiè de l'Ouest, in France.

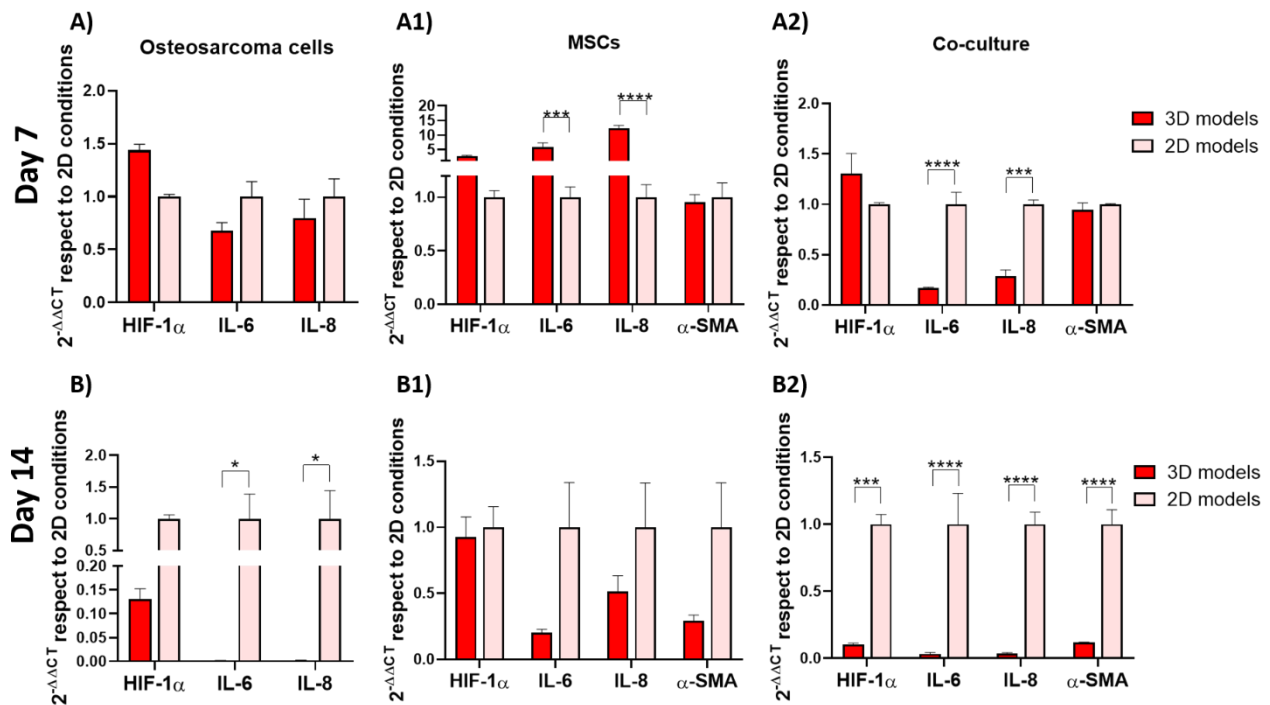


Figure 19. Gene expression of TME markers. Gene expression of HIF-1α, IL-6, IL-8 and α-SMA as fold-change expression relative to 2D conditions in osteosarcoma cells (A, B), MSCs (A1, B1) and co-culture (A2, B2). The graphs show the mean ± SEM. *P value ≤ 0.05, ***p value ≤ 0.001, ****p value ≤ 0.0001.

Finally, the quantification of Metalloproteinases (MMPs) and their related Tissue Inhibitors of MMPs (TIMPs) have been investigated by ELISA kits (Figure 20). The MMPs are a group of zinc and calcium-dependent proteolytic enzymes whose primary role is to hydrolyze the ECM, and TIMPs can inhibit their activity^{318, 319}. In normal tissue, MMPs regulate a series of physiological processes and signal events, such as embryogenesis, tissue remodelling, wound healing and angiogenesis; on the other side, TIMPs are responsible for the preservation of the MMPs homeostasis by regulating their activity in physiological conditions, thus minimizing matrix degradation³¹⁹. As a result, the balance

between MMPs and TIMPs plays a key role in the preservation of tissue homeostasis and in preventing pathological processes³¹⁸.

In tumours, including OS, the MMP/TIMP physiological equilibrium was reported to be shifted toward proteolytic activity and changes in their ratio and expression level may be used as a prognostic factor in tumour development and as a therapeutic target³²⁰. The uncontrolled MMPs activity results in excessive degradation of the matrix and release of growth factors, causing tissue damage and functional alteration as a favourable environment for the formation of the primary tumour³²¹. Different studies reported a positive correlation between elevated MMPs levels within the tumour and cell invasion and metastatization³¹⁹.

In this study, the gelatinase MMP-2 and MMP-9, together with the TIMP-3 and TIMP-4, have been analysed in our models (Figure 20). Among the more than 20 members of the MMP family, MMP-2 and MMP-9 have been extensively studied and their overexpression was shown to be closely related to the invasive potential and metastatization of the osteosarcoma^{322, 323}; among the four TIMP members (TIMP-1, TIMP-2, TIMP-3 and TIMP-4), TIMP-3 is involved in the inhibition of both MMP-2 and MMP-9, whereas TIMP-4 can inhibit MMP-2 catalytic activity³¹⁸.

At first glance, it is possible to observe at both time points a higher level of MMP-2 in all cell cultures in both 3D scaffold-based and 2D conditions, compared to the almost undetectable values of MMP-9, suggesting a significative imbalance towards the enzymatic activity of MMP-2 (Figure 20 A, B, A1, B1, C). Specifically, at both time points, a ratio of MMP-9/MMP-2 close to 0 was reported for the co-culture (0.002 and 0.004 at 7 and 14 days, respectively), the iBM-MSCs (0.000 at both time points) and the osteosarcoma cells (0.000 and 0.001 at 7 and 14 days, respectively) in the presence of the scaffold. A low ratio of MMP-9/MMP-2 was reported also in all cell cultures under 2D conditions at both time points (i.e. 0.000 for all cell cultures at day 7; 0.003, 0.0 and 0.024 for co-culture, iBM-MSCs and osteosarcoma cells, respectively, at 14 days) (Figure 20A, B, A1, B1 and C).

These results overall indicate the imbalanced level of the two enzymes, expressing the almost total absence of MMP-9 compared to the higher levels of MMP-2. This outcome is in line with literature showing the potential connection between the predominance of MMP-2 over MMP-9 with poor response to therapy in OS patients³²⁴; several studies observed a more important role of MMP-2, compared to MMP-9, reporting a strong expression of MMP-2 in different osteosarcoma cell lines, biopsies and xenografts while MMP-9 expression was below the detection limit³²⁵. Moreover, it has been observed the predominant MMP-9 activity in the good responder group of patients compared to the predominant MMP-2 activity in the poor responder group, suggesting that both the loss of MMP-9 and/or the gain of MMP-2 activity contributes to poor response to chemotherapy³²⁶.

Looking in detail at the MMP-2 graphs, it is possible to observe the highest levels of enzyme in iBM-MSCs, compared to osteosarcoma cells and co-culture at day 7 ($p \text{ value} \leq 0.0001$) and compared to osteosarcoma cells at day 14 ($p \text{ value} \leq 0.01$), under 3D scaffold-based conditions (Figure 20A); this trend is shown also in 2D conditions, reporting a significant different MMP-2 level in MSCs compared to osteosarcoma cells and co-culture at day 14 ($p \text{ value} \leq 0.0001$) (Figure 20B). It is well-known that a variety of MMPs are expressed in BM-MSCs^{327, 328}, and among them, high levels of MMP-2 play a decisive role in the migrative potential of BM-MSCs in surmounting ECM barriers such as basement membranes to enter into the blood circulation from the bone marrow during tissue repair and regeneration processes³²⁹. Moreover, it has been reported that the expression of MMPs, including MMP-2, is directly associated with MSC proliferative behaviour³³⁰.

In healthy bone tissue, TIMPs inhibit MMPs by forming a strong noncovalent complex with a 1:1 stoichiometry³³¹. However, in the OS the predominant proteolytic activity over the levels of TIMPs is typically reported as a sign of malignancy³²⁰. Considering this, the specific ratio of MMP-2/TIMP-4, MMP-2/TIMP-3 and MMP-9/TIMP-3 was calculated in this study, reporting overall an unbalanced MMP/TIMP equilibrium in favour of the inhibitory activity which is typically related to a reduced proteolytic activity (Figure 20C)³³¹.

Specifically, our results reported a clear unbalanced between the low levels of MMP-9 and the high inhibitory activity of TIMP-3 in all cell cultures under both 3D scaffold-based and 2D conditions, demonstrating the predominant inhibitory activity which favour ECM deposition³³² (Figure A1, B1, A2, B2 and C). A MMP-9/TIMP-3 ratio equal to 0 was reported in the osteosarcoma cells and iBM-MSCs under both 3D scaffold-based and 2D conditions at both time points; the co-culture reported a ratio of 0.001 at 14 days in 2D conditions, that was slightly improved under 3D scaffold-based conditions showing a ratio of 0.002 and 0.004 at 7 and 14 days of culture, respectively. These results overall demonstrated the inhibition of the proteolytic activity, in all the cell culture conditions, in contrast with the 1:1 stoichiometry ratio of healthy tissue and with the predominant proteolytic activity of tumour tissue.

The situation is similar for MMP-2, whose equilibrium with TIMP-3 and -4 was overall shifted towards the inhibitory activity even if more variable ratios were observed among cell cultures under both 3D scaffold-based and 2D conditions (Figure 20A, B, A2, B2, A3, B3, C). Indeed, while a low ratio of MMP-2/TIMP-3 ratio was reported by osteosarcoma cells under 3D scaffold-based conditions at both time points, (i.e. the ratio of 0.128 and 0.293 at 7 and 14 days, respectively), assessing the predominant inhibitory activity, in the co-culture, a more balanced equilibrium between proteolytic and inhibitory activity was reported in the presence of the scaffold (i.e. the ratio of 0.783 and 1.033 at 7 and 14 days, respectively). In the iBM-MSCs, the MMP-2 reported higher levels compared to

TIMP-3 under both 3D scaffold-based (i.e. the ratio of 6.404 and 2.174 at 7 and 14 days, respectively) and 2D conditions for both the cell culture timing (i.e. the ratio of 0.873 and 10.118 at 7 and 14 days, respectively) (Figure 20A, B, A2, B2, C).

Again, the MMP-2/TIMP-4 ratio overall reported higher levels of inhibitor compared to the enzyme in all cell cultures at both time points in the presence of the scaffold and in 2D conditions, (indicating the predominant inhibitory activity over the proteolytic ones (Figure 20A, B, A3, B3, C).

Our results therefore indicate a dysregulated MMPs/TIMPs balance skewed towards the inhibitors, which is in contrast with most of the evidence on OS microenvironment. Nevertheless, accumulating data demonstrate that TIMPs may play contrasting roles in tumour progression, whereas TIMP-3 can induce apoptosis, TIMP-1, -2 and -4 can protect cells from apoptosis³³³⁻³³⁵. It has been shown that TIMPs regulate cell growth and apoptosis through the paracrine mechanism; for example, TIMP-3 may bind to VEGFR2 and competitively inhibit VEGF binding and subsequent kinase signaling³³⁶. TIMP-1 and TIMP-4 can bind to the CD63 and inhibit both the intrinsic and extrinsic cell death, increasing cellular resistance to drugs³³⁷. Considering this, a deeper investigation of the role of MMPs, TIMPs and their ratio is needed to reach comprehensive conclusions and to understand the impact that the unbalanced MMP/TIMP ratio could have on the three-dimensional microenvironment, compared to 2D conditions.

In conclusion, an *in vitro* 3D co-culture model of OS has been successfully developed and characterized, reporting good cellular interactions and colonization of the MgHA/Coll scaffold, together with changes in the mesenchymal phenotype in the presence of tumour cells and predominant TIMPs levels, demonstrating the impact of the microenvironment in cellular responses. RNA sequencing is needed to explore the mesenchymal phenotype and to uncover the impact of MMP/TIMP ratios in the co-culture. Moreover, the functionalization of the matrix with tumoral molecules and with the cell's compartmentalization into specific areas of the scaffold to improve the triggering of tumoral pathways in the microenvironment is needed. This model overall offers a promising preclinical model useful to mimic the malignant interaction between different cell types of the TME and investigate the role of the ECM.

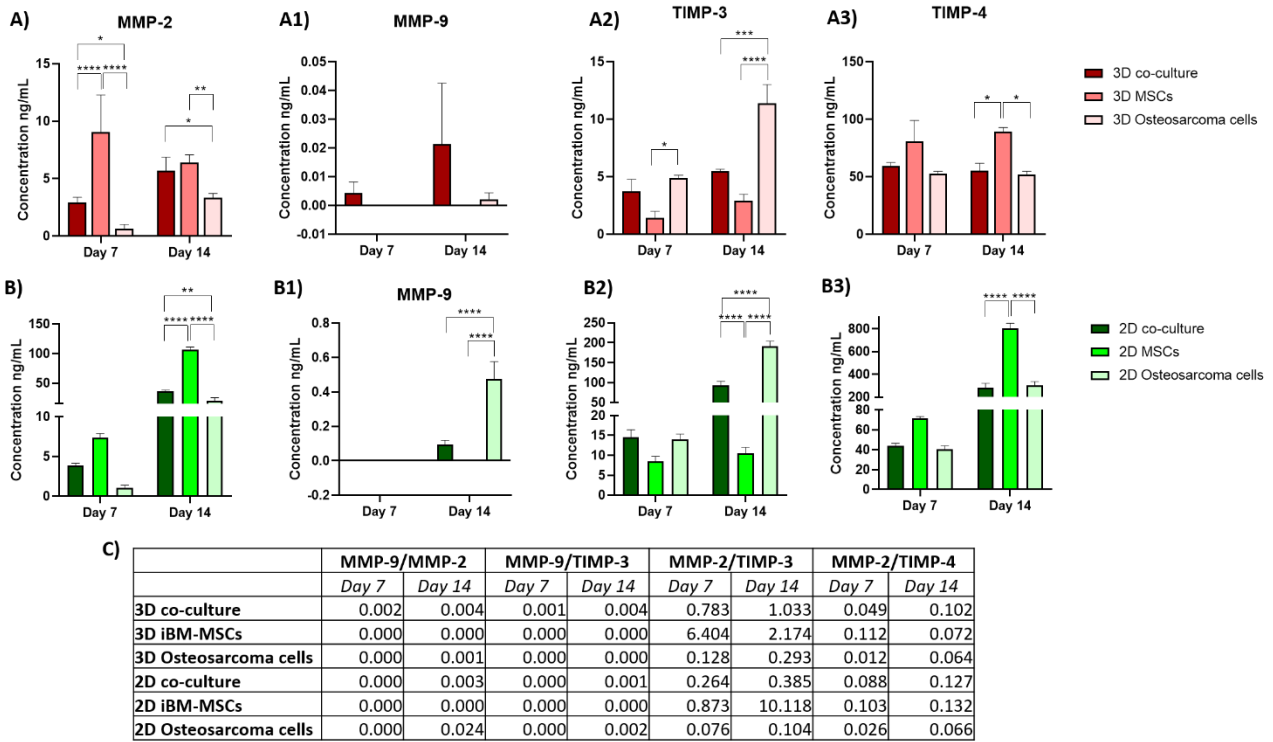


Figure 20. ELISA kits for MMPs and TIMPs quantification. MMP-2 (A, B), MMP-9 (A1, B1), TIMP-3 (A2, B2) and TIMP-4 (A3, B3) in both 3D-scaffold cultures and 2D conditions. The data are reported in the graphs as mean $\pm$ SEM. The MMP-9/MMP-2, MMP-9/TIMP-3, MMP-2/TIMP-3 and MMP-2/TIMP-4 ratios are reported in the table C. *p value ≤ 0.05 , ** p value ≤ 0.01 , *** p value ≤ 0.001 , **** p value ≤ 0.0001 .

5. CONCLUSIONS

In this thesis, various strategies within the 3D Tumour Engineering field were used to recreate a more biomimetic Osteosarcoma (OS) microenvironment, successfully replicating both the structure and function of the tumour mass. Particular attention was given to the critical role of the Cancer Stem Cell (CSC) niche and the reciprocal interactions between the extracellular matrix (ECM) and different cell populations.

The careful selection of sarcospheres, combined with the development of a custom-designed injection method for their precise seeding into the MgHA/Coll scaffold matrix, enabled the creation of a 3D CSC niche model. This model can address the limitations of standard 2D cell culture systems and provides a valuable platform for exploring innovative and efficient therapeutic strategies.

Furthermore, the implementation of a co-culture system, involving both tumour cells and healthy mesenchymal stem cells (MSCs), onto the MgHA/Coll scaffold, resulted in the successful development of a 3D OS co-culture model. This model demonstrated how the interaction between cellular components and the ECM significantly influences the tumour microenvironment (TME) and subsequent cellular responses. This system can serve as a foundational tool for investigating the dynamic interactions between tumour and stromal cells.

In conclusion, the two proposed 3D OS models represent innovative approaches for mimicking tumour behaviour. These systems offer a platform for addressing fundamental questions in cancer biology and fostering interdisciplinary collaboration to better understand the heterogeneity of OS, ultimately advancing the field of tumour engineering and therapeutic research.

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Main Activities

Research period off site and abroad

01/04/2024 – 31/05/2024. Research period off-site (Two months). At: Centre for Nanotechnology Innovation@NEST, Istituto Italiano di Tecnologia (IIT), Pisa, Italy. Funded by: PREDICTOS HORIZON-WIDERA-2021-ACCESS-03-01-TWINNING, grant n. 101079372. Research activity: *In vivo* grafting of 3D CSCs niche model of osteosarcoma in CAM model for drug resistance evaluation.

16/03/2023. Research day off-site. Research meeting at the Centre for Nanotechnology Innovation@NEST, Istituto Italiano di Tecnologia (IIT), Pisa, Italy. Activity: *in vivo* grafting of 3D CSCs niche model of osteosarcoma into CAM model.

06/11/2022 – 27/11/2022. Short Term Mobility Program 2022 (One month). At: Institut de Cancerologie de l'Ouest, Nantes, France. Research group “tumour heterogeneity and personalized medicine”. Founded by SAC.AD002.045 Programma Short Term Mobility (STM) - GAE P0000148 Bando Programma Short Term Mobility. Research activity: Development of 3D models of co-culture of osteosarcoma *in vitro*.

03/10/2022 – 10/10/2022. Short Term Exchange Nano4tarmed.

At: Palacký University of Olomouc, Repubblica Ceca. Funded by NANO4TARMED H2020-WIDESPREAD-2020-5-952063. Grant agreement No. 952063. Research activity: Development of 3D scaffold-based systems *in vitro* for drug and nanoparticles testing.

Congresses

Networking conference for the 10 Years MERLN Institute, Maastricht, The Netherlands, from the 16/10/24 to the 18/10/24. Poster presentation titled “In vitro and In vivo Drug Resistance evaluation of 3D Osteosarcoma Models of Cancer Stem Cells niche as advanced predictive platform”. Authors: Giada Bassi, Arianna Rossi, Mohamed Saqawa, Valerio Voliani and Monica Montesi. Poster presentation titled “Development of 3D Advanced Predictive Models of Osteosarcoma for the investigation of therapeutic mechanisms”. Authors: Giada Bassi, Arianna Rossi, Mohamed Saqawa, Silvia Panseri and Monica Montesi.

SIB (Società Italiana Biomateriali), Faenza, from the 8/07/24 to the 10/07/24. Oral Presentation titled “3D Tumour Engineered Models of Osteosarcoma Stem Cell Niche: in vitro and in vitro Drug Resistance evaluation”. Authors: Giada Bassi, Arianna Ross, Mohamed Saqawa, Lorenzo Apolloni, Elisabetta Campodoni, Monica Sandri, Patrizia Sarogni, Valerio Voliani, Silvia Panseri and Monica Montesi. Fire Poster presentation titled “Enhancing excellence for the development of 3D advanced predictive models of osteosarcoma for the investigation of therapeutic mechanisms”. Authors: Giada Bassi, Mohamed Saqawa, Diana Pacheco, Alexandre Gomes, Marta Lourenço, Artur Mateus, Nuno Alves, Silvia Panseri, Tatiana MF Patrício, Monica Montesi.

TERMIS WC congress, Seattle, Washington USA, from the 24/06/24 to the 29/06/24. Poster presentation titled “Reciprocal Interactions between Osteosarcoma Cells and Mesenchymal Stem Cells: exploring in vitro

Co-Culture Models in 2D and 3D Scaffold Environment”. Authors: Giada Bassi, Arianna Rossi, Mohamed Saqawa, Elisabetta Campodoni, Monica Sandri, Denis Cochonneau, Dominique Heymann, Stefania Fulle, Monica Montesi. Poster presentation titled “Enhancing excellence for the development of advanced predictive and therapeutic models for osteosarcoma”. Authors: Giada Bassi, Mohamed Saqawa, Diana Pacheco, Alexandre Gomes, Marta Lourenço, Artur Mateus, Nuno Alves, Silvia Panseri, Tatiana MF Patrício, Monica Montesi.

EuSARC congress, Le Pouliguen, France, from the 13/06/24 to the 15/06/24. Flash oral presentation titled “Advanced Tumour Engineered 3D Models of Osteosarcoma as in vitro predictive preclinical platforms”. Authors: Giada Bassi, Arianna Rossi, Mohamed Saqawa, Elisabetta Campodoni, Monica Sandri, Patrizia Sarogni, Valerio Voliani, Denis Cochonneau, Dominique Heymann, Stefania Fulle, Silvia Panseri and Monica Montesi. Poster presentation titled “Enhancing excellence for the design, development and investigation of advanced predictive and therapeutic osteosarcoma models”. Authors: Giada Bassi, Mohamed Saqawa, Diana Pacheco, Alexandre Gomes, Marta Lourenço, Artur Mateus, Nuno Alves, Silvia Panseri, Tatiana MF Patrício, Monica Montesi.

TERMIS EU chapter, Manchester UK, from the 27/03/2023 to the 31/03/2023. Oral presentation titled “In vitro 3D osteosarcoma models of CSC-niche: optimization of serial sarcospheres and scaffold-based seeding as cancer-specific instructive microenvironment”. Authors: Giada Bassi, Silvia Panseri, Arianna Rossi, Elisabetta Campodoni, Monica Sandri, Stefania Fulle, Monica Montesi. Poster presentation titled “2D and 3D in vitro study of novel Pt(IV)-Carbohydrate Derivatives as Targeted Anticancer Agents against Osteosarcoma”. Authors: Giada Bassi, Eoin Moynihan, Arianna Rossi, Elisabetta Campodoni, Andrea Ruffini, Eithne Dempsey, Silvia Panseri, Trinidad Velasco-Torrijos, Diego Montagner, Monica Montesi.

Bioceramics 32nd, Venezia Mestre, Italy, from 20/09/22 to 23/09/22. Poster presentation titled “3D Bioceramic scaffolds as cancer-specific instructive microenvironment”. Authors: Giada Bassi, Silvia Panseri, Arianna Rossi, Elisabetta Campodoni, Monica Sandri, Massimiliano Dapporto, Simone Sprio, Anna Tampieri, Stefania Fulle, Monica Montesi. The poster won the Best Poster Presentation award funded by JECS Trust ECerS. Poster presentation titled “Deciphering the interactions of magnetic Iron-doped Hydroxyapatite Nanoparticles with bone cells”. Authors: Giada Bassi, Arianna Rossi, Silvia Panseri, Elisabetta Campodoni, Alessio Adamiano, Monica Sandri, Anna Tampieri, Monica Montesi.

Scientific publications related to the PhD project

Bassi G, Rossi A, Campodoni E, Sandri M, Sarogni P, Fulle S, Voliani V, Panseri S, Montesi M. 3D Tumor-Engineered Model Replicating the Osteosarcoma Stem Cell Niche and In Vivo Tumor Complexity. ACS Appl Mater Interfaces. 2024 Oct 1. doi: 10.1021/acsami.4c02567. Epub ahead of print. PMID: 39353598.

Impact factor: 9.5

Scientific publications related to other collaborations (from the most recent to the least recent)

Rossi A, Bassi G, Cunha C, Baldisserri C, Ravaglia N, Gardini D, Molinari F, Lista F, Teran FJ, Piperno A, Montesi M, Panseri S. Magnetically induced anisotropic structure in an injectable hydrogel for skeletal muscle regeneration. *J Colloid Interface Sci.* 2024 Sep 16;678(Pt C):334-345. doi: 10.1016/j.jcis.2024.09.121. Epub ahead of print. PMID: 39298986.

Impact factor: 9.4

Degli Esposti L, Squitieri D, Fusacchia C, Bassi G, Torelli R, Altamura D, Manicone E, Panseri S, Adamiano A, Giannini C, Montesi M, Bugli F, Iafisco M. Bioinspired oriented calcium phosphate nanocrystal arrays with bactericidal and osteogenic properties. *Acta Biomater.* 2024 Sep 15;186:470-488. doi: 10.1016/j.actbio.2024.08.001. Epub 2024 Aug 6. PMID: 39117114.

Impact factor: 9.4

Rossi A, Furlani F, Bassi G, Cunha C, Lunghi A, Molinari F, Teran FJ, Lista F, Bianchi M, Piperno A, Montesi M, Panseri S. Contactless magnetically responsive injectable hydrogel for aligned tissue regeneration. *Mater Today Bio.* 2024 Jun 3;27:101110. doi: 10.1016/j.mtbio.2024.101110. PMID: 39211510; PMCID: PMC11360152.

Impact factor: 8.7

A. Rossi, C. Stagno, A. Piperno, N. Iraci, S. Panseri, M. Montesi, M. Feizi-Dehneyebi, G. Bassi, M. L. Di Pietro, N. Micale, *Appl Organomet Chem* 2024, 38(5), e7403. <https://doi.org/10.1002/aoc.7403>.

Impact factor: 3.7

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