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Mesenchymal Stem Cells Relevance in Multicellular Bioengineered 3D *In vitro* Tumor Models

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Abbreviations: AT-MSC's, Adipose Tissue Stem Cells; **BM-**MSC's, Bone Marrow Mesenchymal Stem Cells; **bFGF**, Basic Fibroblast Growth Factor; **CAF**, Cancer Associated Fibroblasts; **CD**, Cluster of Differentiation; **CSC's**, Cancer Stem Cells; **EC,** Endothelial Cells; **ECM,** Extra-Cellular Matrix; **EMT**, Epithelial to Mesenchymal Transition; **EGF**, Epidermal Growth Factor; **FGF**, Fibroblast Growth Factor; **GFP**, Green Fluorescent Protein; **HGF**, Hepatocyte Growth Factor; **IL**, Interleukin; **IGF,** Insulin Growth Factor; **IGFBP**, Insulin Growth Factor Binding Proteins; **L-CSCs**, Lung Cancer Stem Cells; **MAPK**, Mitogen-activated

Protein Kinases; **MCP**, Monocyte Chemoattractant Protein; **MHC-II**, Major Histocompatibility Complex II; **MM**, Multiple Myeloma; **MSC's**, Mesenchymal Stem Cells; **MSC-CC,** Mesenchymal Stem Cell to Cancer Cells; **PDGF**, Platelet-Derived Growth Factor; **RFP**, Red Fluorescent Protein; **SDF** , Stromal Cell-Derived Factor; **TA-MSC's**, Tumor- Associated Mesenchymal Stem Cells; **TGF**, Tumor Growth Factor; **TME**, Tumor Microenvironment; **TNF,** Tumor Necrosis Factor**; TREAT-ME1,** Treatment of Advanced Gastrointestinal Tumors with Genetically Modified Autologous Mesenchymal Stromal Cells; **VEGF**, Vascular Endothelial Growth Factor; **WJ-MSC's**, Wharton Jelly Mesenchymal Stem Cells; **3D**, three-dimensional; **3D MCTS**, 3D Multicellular Tumor Spheroids;

Abstract

In vitro 3D tumor microenvironment mimicking models are gathering momentum as alternatives to traditional 2D flat monolayer cultures due to their potential for recapitulating major cancer hallmarks. To fulfill such potential, it is crucial that 3D tumor testing platforms completely emulate *in vitro* the complex *in vivo* tumor niche and its cellular constituents. Mesenchymal stem cells (MSCs) are recognized to play a pivotal multi- modulatory role in cancer, generating interest as biological targets and as key tumor suppressing, or tumor promoting effectors. This review discusses the biological influence of different types of MSCs in the tumor microenvironment and showcases recent studies that engineer 3D MSCs-cancer cells co-cultures as advanced *in vitro* therapy testing platforms. A special focus is given to MSCs-Cancer 3D co-culture set-up parameters, challenges, and future opportunities. Understanding cancer-MSCs crosstalk and their underlying effects is envisioned to support the development of advanced 3D *in vitro* disease models for discovery of forefront cancer treatments.

1 Introduction

In vitro pre-clinical cancer models are mainly based on the use of 2D cancer cell monolayers and laboratory animal models [1,2]. Historically, both methodologies have been recommended by regulatory agencies to aid in the discovery and validation of anti-cancer therapies that are to be administered in human clinical trials [1,3]. However, while these platforms have contributed immensely to explore cancer development and biomarkers discovery, they are still inadequate approximations of human tumors [4,5].

Particularly, in 2D *in vitro* flat and monotypic cell culture models, cancer cells experience highly artificial environments often comprised of plastic or glass cell-adhesive surfaces, forcing cells to grow in an environment which lacks major extracellular matrix (ECM) and stromal cell components [6]. As a consequence, cultured cancer cells are exposed to an unnatural 2D spatial organization and non-physiological conditions, presenting a higher area of exchange with cell culture media in comparison to what naturally occurs in 3D tissues *in vivo* [6]. Such results in an abnormal morphology, loss of structural organization and cell polarization, with lower cell adhesion occurring for example due to loss of integrin- ECM interactions [7]. Therefore, cells phenotype and their response to different treatments is not representative of complex human tumors, thus creating a gap between *in vitro/in vivo* data correlation.

On the other hand, despite animal models constitute a more laborious and economically demanding alternative, they are more representative of the *in vivo* scenario than conventional 2D *in vitro* cell cultures [8]. A significant number of early stage preclinical *in vivo* drug screening studies are performed in small animal murine models. However, these often lack a correct representation of the tumor stroma and present expression variances in the structural homology of molecular targets, which can result in highly variable therapeutic responses [8-11]. Such variability is somewhat detrimental for the validation of candidate anti-cancer therapeutics and particularly limiting in the case of combinatorial cancer therapies high-throughput screening. Such treatment modality demands a higher level of reproducibility and predictability of safety/efficacy parameters as the cocktails of bioactive molecules could trigger a

wide range of biological responses (e.g., additive toxicity, antagonism). The use of *in vivo* models in the context of combinatorial anti-cancer therapies entails significant ethical concerns due to the large number of test groups, not easily allowing high-throughput screening of different combinations. In addition, the correlation between *in vivo* animal studies and human clinical trials is very limited, thus evidencing the necessity of developing more realistic testing platforms that can provide robust preclinical data [8,9,12].

Hence, the development of *in* vivo-mimicking, and reproducible preclinical *in vitro* validation models to efficiently predict the biological performance of anti-cancer therapies may contribute to increase the translation speed of novel therapies from the bench-to- bedside. The bioengineering of such models must be based on similarities to human biology and disease specific features, so as to ensure higher predictability of preclinical research and exclude non-adequate anti-cancer therapeutic candidates prior to human clinical trials [3,13].

The demand for understanding cancer development and develop more advanced treatments has resulted on a growing number of studies exploring the potential of advanced three-dimensional (3D) tumor models as more viable testing platforms. 3D multicellular tumor models are generally self-assembled compact cellular agglomerates that may be cultured *in vitro* during relatively short or prolonged periods of time using different techniques that include hanging drop, bioreactors or organ-on-a-chip platforms [14,15]. Such platforms try to incorporate the spatial complexity, cellular heterogeneity, nutrient/pH characteristic of the *in vivo* tumor microenvironment [14]. In this context various types of malignant cells were shown to possess gene expression patterns and phenotypes similar to those encountered *in vivo* when cultured in 3D [16]. Such evidence is an added-value to these models, since on one hand they provide the desired spatial distribution and on the other they ensure several biological functions found in human cancers [17].

This review summarizes the recent advances made in the field of 3D *in vitro* multicellular tumor models that more closely recapitulate the tumor microenvironment and its diverse components by including co-cultured cancer-stromal cells, including mesenchymal stem cells (MSCs). MSCs are now well-recognized to take multi-modal roles in oncological disease progression and metastasis, for which

depending on MSCs tissue of origin and the type of tumor, they either play a beneficial or detrimental role [18]. This interesting duality is discussed considering the most recent literature reports and a critical perspective is given towards the development of MSCs-Cancer 3D *in vitro* co-culture tumor models.

2 The Dual Role of Mesenchymal Stem Cells in the Heterogeneous 3D Tumor Microenvironment

Mesenchymal stem cells (MSC) otherwise also known as multipotent mesenchymal stromal cells, were first described as fibroblast-like cells, about 50 years ago [19]. They are a subset of multipotent precursor stromal cells, with fibroblast-like morphology. MSCs represent a cell sub-population that can be found residing in the mesenchyme of a wide variety of tissues, including: (i) umbilical cord Wharton jelly, (ii) placenta, (iii) peripheral and fetal blood, (iv) adipose tissues (generally defined as adipose-derived stem cells (AT- MSCs)), (v) skeletal muscle, (vi) heart, (vii) liver, (viii) lung tissues and(iv) bone marrow [20]. Constituting a heterogeneous cellular population, MSCs have diverse morphologies, and are commonly identified through specific cell surface markers. Under the guidelines issued by the International Society of Cellular Therapy, MSCs in *in vitro* cultures must be able to adhere to plastic treated surfaces in standard tissue culture conditions; with more than 95% of the population expressing CD105, CD90, CD73, and with less than 2% presenting positive CD45, CD14, CD34 or CD11b, CD79a or CD19 and major histocompatibility complex II (MHC-II) markers. Besides this, *in vitro* cultured MSCs must also show multilineage differentiation capacity. MSCs traditionally differentiate into chondrocytes, adipocytes and osteoblastic lineages under controlled *in vitro* differentiating conditions [21]. A small sub-population of bone-marrow derived MSCs may still be negative for CD44, CD45, MHC I, MHC II and c-kit, displaying the capacity to differentiate as well into nerve, pancreas and lung cells under certain culture conditions [22].

MSCs are reported to exhibit immunosuppressive and immunomodulatory properties [23], as well as being able to migrate and induce modifications in damaged or inflamed tissues [24]. These abilities are normally explored under the context of cell-based therapies for tissue repair and regeneration, either via direct cell-cell contact or by means of paracrine signaling [24]. MSCs are also described to have high

tumor tropism, a process that is generally mediated by chemokines and growth factors [25]. Upon migrating into the tumor microenvironment (TME) and establishing contact with this complex, MSCs can be hijacked by cancer cells, either through cell-cell or paracrine interactions, leading to either beneficial or detrimental roles in the evolution of cancer [26]. For instance, MSCs recruited to the TME of breast cancers have shown to possess increased secretion of neovascularization and epithelial-mesenchymal transition (EMT) promoting factors, such as Notch1 and TGF-^1, thus promoting tumor growth and metastasis [27-29]. Positive interactions between cancer cells and MSCs are not restricted to a specific phenotype of MSCs. For example, umbilical cord derived MSCs have shown to greatly increase cholangiocarcinoma proliferation, migration and resistance (Figure 1) [30]. MSCs can also indirectly aid the evolution of cancer by regulating the activity of other TME cells [25]. For example, breast cancer-associated bone-marrow MSCs (BM-MSCs) are known to increase the frequency of regulatory T cells, and decreasing the lysis activity of natural killer cells and cytotoxic T lymphocytes by the secretion of TGF-^1, resulting in a poorer prognosis due to unchecked tumor progression [30,31].

Contrariwise, MSCs have also been reported to exert anti-tumor effects [32], with several studies having demonstrated the role of MSCs in increasing the growth and metastasis of tumors [18,33]. In an elegant a study performed by Quiao and co-workers, 2008, MSCs derived from fetal dermal tissue where shown to inhibit the proliferation of two human hepatocarcinoma cell lines (H7402, HepG2), when co-cultured [34]. Such resulted in an increased apoptosis for H7402 cells via the down regulation of the Wnt/p-catenin pathway as proposed by these researchers [34].

Interestingly, it is important to emphasize that this effect is closely correlated with MSCs tissue of origin (Figure 2). As reported by Attar-Schneider *et al,* 2015, BM-MSCs can reduce both cellular proliferation, viability and migration of non-small lung cancer cells as a result of the down regulation of translation initiation factors and mitogen-activated protein kinases (MAPK) signaling [35]. However, the same is not true for Wharton jelly-derived MSCs which show antagonistic effects to those portrayed by BM-MSCs in lung cancer stem cells (L-CSCs), acting in a pro-tumorigenic manner [32]. Conversely, conditioned media derived from human fetal MSCs showed high levels of insulin growth factor binding

proteins (IGFBP), which when used in hepatocellular carcinoma cell culture, ends up sequestering free IGF, inhibiting cancer cell proliferation [36]. Due to this anti-proliferative potential, MSCs have been proposed to be used as Trojan-horse like vehicles for anti-cancer therapy by taking advantage of their tropism towards cancer cells [37]. By exploiting this feature, TREAT-ME1, the first worldwide clinical trial exploring the use of Herpes simplex virus genetically engineered MSCs for treatment of gastrointestinal tumors via RANTES/CCL5- ganciclovir prodrug therapy combination [25]. However, the lack of knowledge on how MSCs behave within the TME means that patients undergoing these treatments must be carefully monitored. Thus, understanding how MSCs from different tissue origins affect, and

are affected, by tumors is of paramount importance for future therapeutic applications (Figure 2).

2.1. MSCs Tropism towards the Tumor Microenvironment

Mesenchymal stromal cells interactions with cancer cells and the tumor microenvironment result in part from the similarities existent between the TME and those of a regenerating wound [18]. Generally, the TME continuously produces and releases various cytokines and other signaling mediators, that act as chemoattractants for several immune cells such as monocytes, T lymphocytes and mast cells. This establishes the grounds for a perpetual state of inflammation in the surrounding tissues [38,39]. Such signals derived from the tumor microenvironment, include: VEGF, TGF-pi, EGF, HGF, bFGF, PDGF, IL-8, neurotrophin-3, IL-ip, TNF-a, monocyte chemoattractant protein-1 (MCP-1 or CCL2), and C-X-C motif chemokine ligand 12 (CXCL12) also known as stromal cell-derived factor 1 (SDF-1) which act as homing beacons on various cells including MSCs which exhibit the capacity to accurately migrate via chemotaxis [33]. This tumor tropism towards primary and metastatic tumor sites by MSCs has been extensively observed both *in vivo* and *in vitro* in numerous cancers, such as those of breast, brain, colon, pancreas, skin, ovarian, and lung, and is characterized by MSCs infiltration [26,33]. The extent of MSCs migration appears to be directly influenced by the extent of chemoattractant signals released by the TME. Recent reports emphasize that factors such as increased inflammation, hypoxia and cell dead (related or not to tumor treatment), increase MSCs migration to tumor tissues [30]. Such event is for

example observed in radiotherapy treated breast cancer, which results in an increased release of release of TGF-pi and platelet-derived growth factor BB (PDGF-BB) by tumor cells [40]. The underlying mechanism responsible for the tumor-directed migratory capacity of MSCs remains however to be fully uncovered. This is a crucial piece of information that must be explored in the future, before MSCs-based treatments can be translated into clinical practice. [26].

2.2. MSCs conversion to Tumor-associated MSCs - Role in Progression and Metastasis

Upon infiltrating on the TME, MSCs are converted into tumor-associated mesenchymal stem cells (TA-MSCs) (Figure 3). Depending on their original tissue, and on the type of cancer, TA-MSCs are known to acquire gene-expression profiles that exhibit increased secretion of tumor promoting factors [41]. TA-MSCs populations have been reported to increase the population of cancer stem cells (CSCs), and induce malignant cells to enter EMT, thereby promoting enhanced motility, invasiveness and survival [41]. In turn, this can induce a refractory profile in the tumor, a factor that can lead to the establishment of metastasis [42]. TA-MSCs have been found to engage in multiple interactions with cancer cells [41]; besides paracrine signaling, communication has also been achieved through the exchange of exosome cell-derived vesicles [43], mitochondria [44] and also cell membrane components [45]. Interestingly, these interactions have been reported to occur in a bidirectional mode. It is important to emphasize that the effects of the exchanged molecules vary with the type of cancer and MSCs population [25]. As previously stated, EMT requires the formation of a reactive stroma, capable of secreting specific EMT-inducing factors and rearranging the surrounding tumor-specific ECM. Various factors that promote the appearance of this reactive stroma such as fibroblast growth factor (FGF), hepatocyte growth factor (HGF), epidermal growth factor (EGF) are produced by MSCs upon impregnation into the tumor niche [33,46]. Some studies point out that this conversion from regular MSCs to TA-MSCs can also be performed *in vitro* through 2D co-culture of TA-MSCs with MSCs, indicating that naive MSC conversion can be augmented once in vicinity of the TME [41].

Besides directly increasing cancer cells EMT, MSCs also increase vasculogenesis or increase the secretion of growth factors, creating a suitable environment to support disease progression [47][33].

Moreover, as aforementioned, MSCs are capable of modulating the metabolism of cancer cells through the excretion of exosomes generally loaded with proteins, DNA, and non-coding RNA (ncRNA) [43]. This effect can be particularly seen in the interactions that occur when cancer cells metastasize into the bone-marrow, a process in which MSCs secrete chemoattractant factors recognized by cancer cells [41]. In this fatal alliance between metastatic cancer cells and MSCs, factors such as MCP-1 (CCL2) and SDF- 1 (CXCL12) appear to play a key role [26,33]. Once metastasis are established in the marrow, MSCs are hijacked into helping in the creation of a niche that protects the tumor against chemotherapeutic agents [18,25,48].

In summary, several factors affect MSCs-cancer cells interactions and MSCs TME interactions, including: (i) the type and source of MSCs including their different cytokines expression (ii) the interactions with other elements of the TME; and (iv) the *in vitro* and *in vivo* conditions in which MSCs are cultured [49,50]. The nature of these interactions and how they take place, either by direct or indirect interference with cancer cells and surrounding stroma, requires further exploration in the future. With such knowledge holding great promise in the context of new therapies discovery, both at the development level by facilitating the discovery of key therapeutic targets, and at the drug-screening stage by allowing the development of more realistic *in vitro* 3D tumors.

3. *In vitro* 3D Tumor models to test candidate anti-cancer therapies

3D cell cultures of cancer cells take aim to recapitulate *in vitro* the complexity of human cancer allowing for better preclinical analysis of new pharmaceuticals, with the goals of permitting the evaluation of possible side effects, and the collection of meaningful compound behavior data before proceeding to human clinical trials [51]. When successfully engineered, 3D *in vitro* tumor models provide a more robust correlation with the *in vivo* biological performance of candidate compounds [52]. In order to fully mimic human tumors, the ideal *in vitro* 3D cancer culture model should reproduce *in vitro* all the interactions and selective pressures that occur in the human body. Unfortunately, the complexity of this task is tremendous and as such, a balance must be found between the model's capacity

to faithfully recreate the TME, while assuring ease of analysis and lowering production costs to efficiently allow high-throughput screening methodologies.

3D models production methodologies extend over a gamut of techniques which can be grouped into: (i) scaffold-free, (ii) scaffold-based and (iii) combinatorial methodologies. Regardless of the production methodology, all these platforms have as a unifying element: the establishment of 3D multicellular structures, or microtissues, comprised of one or more cell types, either derived from immortalized cancer cells cultures or from patient's primary cells [4,52-54]. 3D cell culture methods, range from monotypic cell line cellular spheroids (single cell type), for instance cultured in non-adherent substrates or through hanging-drop techniques [55], up to complex co-culture systems comprising heterotypic cell lines (e.g., tumor stroma cells including fibroblasts) in a complex tumor ECM-like supporting matrix, or even associated with microfluidic systems for dynamic nutrient perfusion [56].

3D Multicellular tumor spheroids (3D MCTS) are seen as gold standard bioengineered microtissues for drug screening [57]. 3D MCTS are generally comprised by an actively proliferating outer cell layer and a necrotic core [58,59]. Moreover, upon aggregation into spherical structures MCTS acquire nutrient, pH, and oxygen gradients ranging from spheroids periphery to their core [59]. These characteristics are in accordance with those of solid tumors with more than 400-600 pm [60][61]. In these models, the lack of vasculature, similar to that of avascular solid tumors, and the establishment of a compact cell aggregate, hinders the mass transport of nutrients, metabolites and oxygen [59]. Additionally, their 3D nature confers them the ability to portray cell-cell biochemical interactions, tumor gene expression patterns, and even growth kinetics similar to those observed *in vivo* [59]. It has also been reported that 3D MCTS models promote cancer cells genetic/epigenetic modifications towards a more aggressive, tumorigenic and multi-drug resistant phenotype [62,63]. Apart from these parameters, heterotypic 3D MCTS (resulting from co-culture of cancer-stromal cells), are able to mimic the deposition of ECM-components similar to those found in solid tumors [57,59,64]. An important parameter, since the ECM-based barrier that hinders O2 and nutrients mass transfer also restricts the penetration of therapeutic agents in the tumor. The existence of ECM creates a suitable *in vitro* environment for testing and

studying new pharmacological therapies and mimics *in vivo* conditions [65]. This correlation, resulting from the incorporation of diverse cell lines, is also observed in scaffold-based models that provide an ECM-mimicking environment without requiring previous cell mediated ECM deposition [64]. The advantages and disadvantages of various 3D models production methodologies have been extensively discussed in several reviews [51,54,56,59,66-71]. These different formulation technologies are addressed herein mainly in the context of heterotypic cancer-MSCs 3D *in vitro* models establishment.

4 Cancer-MSCs Co-culture Relevance in 3D *In vitro* tumor models

Irrespectively of 3D disease models production methodology, heterotypic cell co-cultures comprising cancer-stromal cells provide a better reproduction of the complex tumor microenvironment when compared to monotypic 3D models (comprised by cancer cells alone) [72]. Different bi- or tri- co-culture systems combining cancer cells and stromal cells have thus far been employed for the study of cancer-TME specific interactions and drug screenings [51,54,59].

Recent findings regarding 3D co-culture of cancer-adipose cells, cancer-immune cells, cancer-fibroblasts, cancer-endothelial cells and their physiologically relevant interactions have been reviewed in detail [54]. Stemming from this dual and multiple- cell culture concept, MSCs addition in *in vitro* 3D cancer disease models is still far from being fully explored, particularly when considering their important role in both cancer proliferation and metastasis [73]. In fact, having been found to play such an important role in cancer progression and therapy resistance, MSCs must be considered as key elements in the tumor milieu [41]. As such, their inclusion in *in vitro* 3D tumor models is a necessary step to achieve a more predictive model that closely mimics native *in vivo* conditions. So far, few articles have been published concerning MSCs interactions with cancer cells in *in vitro* 3D co-culture tumor models (Table 1), and their main findings will be addressed in the following sections.

4.1. MSCs Influence in Cancer - Dual Co-Culture 3D Models

Regarding the available studies, in most circumstances MSCs exert a positive influence in cancer

progression, either by increasing malignant cells proliferation, migration, invasion, drug resistance and/or cancer stem cell proliferation. Alternatively, only few studies report MSCs as having negative effects in cancer cells [74-77]. The report by Dittmer and coworkers, [74] demonstrated that BM-MSCs can invade MCF-7 and MDA-MB-231 breast cancer 3D aggregates, originating a disorganized structures by disrupting cell-cell adhesion, mainly through E-cadherin cleavage and nuclear translocation, without however increasing EMT and ERK1/2 activity. These researchers used a lower ratio of MSCs to cancer cells (1:500, and 1:1000) when compared to other studies performed by Mcandrews and colleagues (MSCs:Cancer cells, 1:1 ratio[27]), or Zhu and co-workers (MSCs:Cancer cells, 1:2 and 1:5 ratio [78]), in which different ratios were employed to establish the co-culture of MSCs and breast cancer cells in hydrogel-based scaffolds. The data collected by Dittmer, suggests that low numbers of MSCs are capable of increasing breast cancer cell migration [74]. On another report, Klopp and colleagues, 2010, obtained a more organized and spherical 3D model upon inclusion of BM-MSCs when using the same cell lines as models [79]. The authors attributed these differences to the incorporation of MSCs prior to the formation of the aggregates and to the lack of serum-containing media. In a different approach Dittmer also evaluated if the presence of MSCs in MCF-7 and MDA-MB-231 spheroids affected their susceptibility to 4 tyrosine kinase inhibitors. The interesting results that were obtained indicate that in both spheroids (MCF-7:MSCs and MDA-MB-231:MSCs), the effect of the administered therapeutic anti-cancer drugs was augmented, pinpointing the decreased resistance to alterations in ERK1/2 phosphorylation and PKCa [75].

Several subsequent studies [27,78,80,81], using scaffold-based technologies for 3D models assembly provide evidence that BM-MSCs exert a positive effect in cancer cell survival and migratory capacity. In fact, cells co-cultured in tissue mimetic scaffolds including those based on: (i) collagen hydrogels [27]; (ii) silk-fibroin scaffolds [81], or (iii) porous chitosan scaffolds containing hydroxyapatite organotypic as bone mimetics [78] laden with undifferentiated or differentiated MSCs, evidence their capability to up-regulate the expression of EMT-related genes in cancer cells, to induce higher migratory capacity (Figure 4), and to enhance proliferation through different signaling factors. For example through the release of

cyclic AMP [78], and TGF-p phosphorylation [27].

Moreover, MSCs are also able to increase cancer stem cell populations [80] and cancer cells resistance to therapeutics. In a study by Jakubikova and colleagues, the interactions between BM-MSCs and multiple myeloma (MM) where analyzed using a co-culture model derived from cellular populations extracted from patients in different stages of MM [82]. The results obtained by the authors demonstrated that in comparison to MM cells alone, the co-cultured cells exhibited resistance to an extensive panel of pharmacological agents, both novel and conventional, replicating clinical observations (Figure 5).

More recently, the development of an interesting MSCs-cancer cells scaffold-based 3D *in vitro* model for drug screening was also described by Han and co-workers. In this model MSCs and human non-small cell lung carcinoma cells A549 cells where co-cultured in wells coated with a multi-layer film of chitosan and hyaluronan (Figure 6) [83]. In this layer-bylayer structured biomaterial, cancer cells and MSCs efficiently self-organized into spheroids, presenting a core-shell structure wherein A549 cells exhibited enriched 2-fold upregulation of EMT-related genes, in comparison with 2D cultures. This remarkable enrichment was also observed in conditioned medium experiments with 2D cultures exhibiting lower EMT and tumorigenicity associated factors than those of direct co-culture [83]. Interestingly, *in vivo* assays demonstrated that co-culture derived implants presented higher malignancy, therefore highlighting the importance of MSCs cancer cell direct contact in tumor progression.

On the other hand, the establishment of negative interactions between BM-MSCs and cancer cells has been reported in human colon cancer cell lines HT29 and HCT-116, which presented decreased proliferation when co-cultured *in vitro* in a three-dimensional scaffold (of unmentioned composition) with BM-MSCs [76]. In this study BM-MSCs where found to secret PAI-1, a factor closely associated with aggressive colon cancer [84], and showed to have a direct effect in cancer cell proliferation. In the case of HCT-116 cancer cells BM-MSCs excreted PAI-1 was shown to yielded a negative influence in cellular proliferation, while with HT29 this interaction lead to a positive outcome, in which high concentrations of PAI- 1 increased cell proliferation [76]. However, when taking into account all factors secreted by MSCs, these exerted a negative effect resulting in a decrease in cancer cell proliferation in

the order of 50% [84]. This fact becomes extremely relevant when one considers that MSCs of different origins are known to express different levels of cytokines and signaling factors in the same context [85,86], emphasizing once again the necessity of further studying the influence of MSCs from diverse sources. Still regarding human colon cancer Widder and co-workers, 2016, have demonstrated that BM-MSCs were able to increase HCT8 E-cadherin deficient cell line capacity to assemble into 3D spheroids, whilst increasing HCT8 cell proliferation [87]. This elegant study showcased a close relation between HCT8 and MSCs-specific secretoma, in comparison to that of fibroblasts (CCD18Co). Furthermore, differential MSCs and fibroblasts spatial distribution was

observed amongst 3D spheroids, with external deposition in colorectal cancer cell line DLD1 spheroids and with homogeneous internal deposition in colorectal cancer cell line HCT8 spheroids [87]. The remarkable increase in spheroid adhesion provided by MSCs coculture for HCT8 is proposed by the authors to be correlated with a b1-integrin dependent mechanism, and aided through ECM deposition specifically via the production of collagen I, which also highlights the importance of 3D *in vitro* disease models in the context of cancer cell-stromal cell interaction studies.

4.2. MSCs role in Complex Heterotypic Co-cultures and Organotypic Bone-Metastasis Models

Regarding the development of more complex heterotypic co-culture models, the study by Beckermen and colleagues, established a tri-culture spheroid system comprised by MIA- PaCa-2 cells, primary fibroblasts, and HUVEC cells [88]. These spheroids were then immersed in a methylcellulose/collagen solution and transferred to a fibronectin-coated plate previously cultivated with BM-MSCs in order to simulate and analyze MSCs migration towards tumor-associated blood vessels. The authors observed a positive effect on MSCs migration towards the tri-culture spheroids, in which VEGF appeared to be the prevalent factor with PDGF and EGF also displaying significant chemoattractant properties [88]. *In vivo* studies in mice demonstrated the capacity of ectopically injected BM-MSCs of migrating towards xenografts of human pancreatic cancer, in which BM-MSCs lead to an increase in the number of CD31+ early vascular structures, an event that directly correlates with the density of tumor vascularization, as

was observed, with tumors with twice the vascularization of those in obtained in controls [88]. Another tri-culture was performed by Lamichane and co-workers, 2016, in which A549 cells were co-cultured with human pulmonary microvascular endothelial cells and BM-MSCs [89]. The tracking of the different cellular populations was accomplished through transfection with lentiviral particles encoding red fluorescent (RFP) and green fluorescent (GFP) transgene reporters, and followed for 15 days. The results showed a decline in the endothelial population, and an accentuated increase both in MSCs, which primarily formed the core of the spheroid, and cancer cells. From the endothelial cells co-cultured in these spheroids only a small population remained viable, being hypothesized by the authors to have survived by close association with MSCs within the hypoxic regions of the necrotic core. The spheroids exhibited increased multidrug resistance markers expression (ABC-B1 mRNA and ROS). However, no increased drug resistance in the 3D models was obtained when compared to standard 2D co-cultures. In fact, only some concentrations showed a prevailing difference between models, with the 3D model presenting always an increased cellular viability in comparison to the 2D model [89].

The importance of the 3D organization in cancer cell-mesenchymal communication was sophisticatedly addressed in a study performed by Pasquier and co-workers [90]. This report employed both ovarian and breast cancer cell lines and the authors demonstrated the existence of a ubiquitous mechanism of cytoplasmic material transference through the usage of tunneling nanotubules in spatially adjacent cells, using 3D spheroid tri-culture systems [90]. The results indicated that mitochondrial transference between cancer cells and stromal cells took place, preferably, between endothelial cells (EC) and cancer cells, also occurring in the same mode between explant tissue and co-cultured EC cells. This exchange endowed cancer cells with a proliferative advantage by rescuing them from erratic aerobic respiration dysfunctions and endowed them with a significant resistance to chemotherapeutic agents [90]. This report further demonstrates the necessity for analyzing cancer cells-MSCs interactions in a tumor stroma context.

In addition to being used for the establishment of 3D drug testing platforms or in the study of the complex interactions with cancer MSCs have also been employed for establishment of organotypic 3D *in*

vitro bone metastasis models. Such approach takes advantage of the multilineage differentiation potential of BM-MSCs, namely their capacity to differentiate into osteoblasts. In this context, Jeon and colleagues, have developed a collagen I hydrogel laden with BM-MSCs differentiated into osteoblasts co-cultured with vascular endothelial cells (HUVECs) and enclosed it in a microfluidic device (Figure 7) [91]. The co-culture of osteo-differentiated MSCs and HUVECs promoted the formation of endothelium around the microfluidic channels and the deposition of bone-marrow specific biomolecules and matrix. This highly organotypic model was then used to evaluate the behavior of breast cancer cells that were injected in the channels of the hydrogel-containing chip. The obtained results demonstrate that cancer cells in the tri-culture microfluidic model have a higher prometastatic potential, with higher rates of extravasation, migration and invasion. Such increased aggressiveness was evidenced by the breast cancer micro-metastatic pockets containing 4-60 cells that formed at 5 days of co-culture [91]. However, it is important to point out that due to the lack of MSCs population tracking during differentiation into osteoblasts it impossible to establish a direct connection between cells invasiveness and their interactions with possibly present non-osteogenic differentiated MSCs. This evidences the requirement for long-term cell tracking in these *in vitro* models, a parameter that will be further discussed.

4.3. Importance of MSCs Tissue of Origin and Cancer Heterogeneity

It is relevant to highlight that the origin of MSCs may also play an important role in cancer and consequently on the development of more advanced 3D *in vitro* disease models. In fact, a comparison between the effects of co-culturing Wharton Jelly-derived MSCs (WJ-MSCs) or BM-MSCs with lung cancer stem cells, either in 3D using multicellular spheroids or in 2D demonstrated contrasting effects between WJ-MSCs and BM-MSCs towards lung cancer stem cells (LCSC), with different subtypes of LCSC presenting different responses to WJ- MSC, especially when co-cultured in 3D [32]. Tumor heterogeneity may also play a role in cancer-MSCs interactions and hence influence the conclusions extrapolated from *in vitro* models. A recent study by Breznik and co-workers, 2017, demonstrated how phenotypically different cell lines used as models for glyoblastoma can lead to different MSC-cancer

cells interactions and outcomes, with U87 cells showing decreased invasive potential, and U373 cells displaying the opposite, both *in vivo* and *in vitro* [92]. The researchers attributed this difference to alternatively favored metabolic pathways resulting from TGF-p release by MSCs. Thus underlining the importance of accounting for the heterogeneity and phenotypic variations present in actively proliferating tumors [92]. These results emphasize the necessity of considering the use of MSCs from several tissue origins, as well as different types of malignant cells to account for intrinsic *in vivo* tumor cellular heterogeneity.

5 Key Technical Parameters for Establishment of Cancer Cells-MSCs 3D *In vitro* CoCulture Models

The number of variables that can affect the interaction and co-culture of such diverse cell populations makes achieving a valid co-culture model containing MSCs extremely difficult. In this context, it is important to emphasize that the relative lack of knowledge about the exact composition of both non-cellular and cellular components of the TME drastically reduces the ability to implement *in vivo* similar co-culture ratios. In addition, different cancer types are expected to possess diversified cell populations and thus, different ratios, such as those characteristic of brain and NSLC [93,94]. Moreover, variability may derive from different stages of the same cancer as verified in MM by [82]. The creation of *in vivo* mimicking *in vitro* 3D models for the study of cancer cells-MSC interactions have thus far relied in the usage of basic approaches. In a study by Zhu and colleagues, 3 diverse ratios (1:1, 1:2 and 1:5) where used for the co-culture of BM-MSC with breast cancer cells, the authors evaluated MTDH, an oncogene that promotes proliferation and enhances chemoresistance [95], to be up-regulated in co-cultures containing higher ratios of MSC to cancer cells, when compared to lower MSC ratios [78]. In a study by Oerlecke and colleagues, 2013, as few as 1 MSC per 300 breast cancer cell was enough to obtain a significant increase in both Smad3 and CREB phosphorylation in BCC [80]. These findings showcase the necessity of correctly representing the diverse cellular populations of the TME in 3D laboratory models that aim to recapitulate the reality found *in vivo,* since different concentration of MSCs release

dissimilar factors and cytokines that can lead to antagonistic responses [25].

In terms of cell culture specifications, MSCs are known to present diverse media requirements, as well as growth, proliferation, matrix deposition and differentiation capabilities [22] when compared with other cellular components of the TME. With all these processes being affected by the previously referred factors, and by others such as the preexisting scaffolds morphology and composition, which depending on the cell type might induce diversified phenotypes for example amongst cancer cells [96,97]. This complicates the establishment of a heterotypic co-culture model that suits all the necessities of the various cellular populations [98]; especially when modified mediums for the co-culture of cells are still rather underdeveloped [99]. Another problem that arises from these models is how co-culture populations can proliferate/change over extended periods of time, altering the initial experimental parameters as the trial progresses [72]. Such cell modifications can occur, for example, simply through the deposition of extracellular matrix, alteration/degradation of pre-existing scaffolds for 3D culture [54], or in a more complex facet, through the differentiation of the multipotent cells into diverse cellular populations otherwise not accounted for in the model [100]. When accounting for model alterations concerning the co-culture of MSCs and cancer cells, another variance that must be considered is how cancer cell cannibalism towards MSCs may affect the model progression [101]. This phenomenon, was recently addressed by using hanging drop-assembled 3D tumor spheroids which successfully replicated the relatively unstudied process of cellular cannibalism by MDA-MB-231 breast cancer cells (MDA-BCC) directed at MSCs surrounding populations in which cancer cells internalized and consequently degraded MSCs (Figure 8). Such resulted in the acquisition of unique molecular signature profiles by cannibal MDA- BCC cells, presenting an enriched molecular profile for pro-survival factors and tumor suppressor agents, as well as inflammatory mediators which marked these cells as detaining a senescence-associated secretory phenotype [101]. The authors were, thus, able to replicate *in* vitro a process of paramount importance in cancer cell metastasis and therapy survival, where MSCs once again are shown to have a vital role.

Apart from this, the various alterations that cellular populations ratios can suffer during culture must be

carefully monitored, creating a necessity of tracking and analyzing each diversified cellular population in direct contact on 3D co-culture models. To this end, immunostaining techniques can be of use to a certain point, for example when identifying non-stem cell populations or analyzing matrix deposition [102]. Unfortunately, for the differentiation of both cancer stem cells and MSC populations, difficulties might arise due to expression of overlapping receptors or other markers [21,103,104]. The use of cell tracking techniques, such as transfection with non-integrative GFP transgenes via viral or non-viral vectors has been employed as an alternative. However, these methodologies are highly expensive and still present some technical constraints such as transient expression or influence in cells behavior [73,88,105]. Overall, the difficulty of tracking cellular populations hinders the ability to use more complex models for the analysis of specific cellular interactions. Such is the case with explant derived organoids, in which events such as the differentiation of MSCs lineages into osteo-differentiated MSCs must be prevented or dully tagged [106,107].

In summary, the establishment of a 3D MSC-cancer co-culture testing platform encompasses several challenges, namely: (i) different initial seeding ratios can lead to distinct results; (ii) the spatiotemporal manner in which cells are introduced to the model can lead to diverse morphological organizations [79]; with (iii) diverse cells presenting diverse proliferative rates [108]; (iv) *in vitro* cellular division limits (Hayflick limit); (v) and requirement of different types of medium; with all this diversity resulting in the need of (vi) differentially tracking each cell population over time and space.

Conclusions

Upon analysis of different literature reports with 3D cultured MSCs, so far, they mainly focus on the use of co-cultured MSCs-tumor cells 3D spheroids. Only few articles take advantage of scaffold-based approaches and microfluidic chips, but mainly in the study of breast cancer metastasis into bone [78,81,109,110]. Developments in complex scaffold design for tissue engineering could be valuable in producing structures that could provide mechanical/biochemical cues and 3D spatial organization of MSCs and cancer cells [111]. Although the use of simple MSCs-cancer cells 3D co-cultures provides *in vitro* models compatible with high-throughput screening, (one of the main requirements for developing

models for pharmaceutics testing and discovery), several important TME associated factors are disregarded, including tumor-specific ECM, and other stromal cells-cancer cells bidirectional interactions, spatiotemporal mechanical and biochemical cues. Clearly, this lack of ECM and stromal cells representation must be overcome if a representative model is to be achieved, possibly through the usage of complex functionalized scaffolds [112]. These scaffolds can be arranged in various forms in combination with microfluidic chips capable of providing a transport system in which mechanical cues obtained at different perfusion rates, combined with controllable biochemical signals, resembling those of an *in vivo* organism, allowing to increase the complexity of the designed 3D models [98]. It is also important to highlight that the widespread use of BM-MSCs populations in MSCs-cancer cell interactions can result in a bias towards the influence of this particular subset of cells [25,85,86].

In conclusion, further improvements to the currently available 3D *in vitro* tumor models will allow to develop valid platforms that permit the inclusion of MSCs in a way that more closely mimics *in vivo* conditions. While the analysis of individual interactions between these and other cells of the TME can provide a glimpse into the complex weave of communication and mutual regulation that takes place across the intricate setting present in malignant pathologies, ultimately the goal shall be to achieve a model that can be composed of both cancer cells, MSCs and other related stromal cells and immune system cells, in tissue specific ECM-like scaffolds [113]. This will require a combinatorial approach of current technologies, both in the fields of biomaterials and bioengineering [99], in an effort to provide laboratory models that can be used for widespread screening of anticancer therapeutics in a number of different cancer types.

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Conflict of interest

The authors disclose that they have no conflict of interest.

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Table 1. Literature reports of co-culture human mesenchymal stem cells with cancer cells in a 3D co-culture environment.

Type of MSC	Cancer Cell Line used	*In vitro* Model		Tested Therapeutics	Ref.
BM-MSC	Human Pancreatic Cancer - MIA-Pa-Ca2	Spheroids (Formed by MIA-PaCa-2 cells, primary fibroblasts, and HUVEC)	Forced Floating 96 multiwell plates with nonadherent round bottom	None	[88]
BM-MSC	Human Breast cancer MCF7 - MDA-MB-231	Spheroids	Forced Floating Wells Coated with 2% GTG agarose without matrix proteins	None	[74]
BM-MSC	Human Breast Cancer - MDA-ICB-3 - MCF-7 - SUM149	Spheroids	Forced Floating Ultra-low attachment Plates	None	[79]
BM-MSC	Human Breast Cancer - MCF-7 - MDA-MB-231	Spheroids	Forced Floating Wells Coated with 2% GTG agarose without matrix proteins	Kinase inhibitors (TKI258, RAD001 and RAF265)	[75]
AM-MSC	Human Ovarian Cancer - OVCAR 3 -SKOV 3	Spheroids / Scaffold	Forced Floating Ultralow attachment 48 well plate and posteriorly cultured in Amniochorionic Membrane containing MSC	None	[114]
BM-MSC	Human Colon Cancer - HT29 - HCT-116	Scaffold	Cell Culture 3D Scaffold from 3D Biotek LLC	None	[76]
AT-MSC	Human Head and Neck Squamous Cell Cancer (HNSCC) - HLaC78	Spheroids	Forced Floating 96-multiwell plates coated with 0.1% agar	None	[42]
BM-MSC	Human Ovarian Cancer - SKOV3 (HTB-77) - OVCAR3 (HTB-161) | Human Breast Cancer MDA-MB231 - MCF7	Spheroids	Forced floating Ultralow attachment 24 well plate	Doxorubicin	[90]
BM-MSC	Human Breast Cancer - MDA-MB-231	Spheroids	Forced floating Wells coated with 2% SeaKem® GTG agarose without MP	None	[80]
BM-MSC	Human Prostate Cancer - LNCaP - C4-2 - PC3	Agitation Based	Rotating Wall Vessel (RWV)	None	[115]

BM-MSC	Human Breast Cancer - MDA-MB-231 | Human Osteosarcoma - MG-63	Scaffold	Silk protein fibroin scaffold	Paclitaxel	[81]
BM-MSC	Human Leukaemia - HL- 60 - Kasumi-1 - MV411	Scaffold	PGA/PLLA 90/10 copolymer discs	Doxorubicin or Cytarabine	[116]
PG-MSC	Human Prostate Cancer - LNCaP	Spheroids	Formed either in direct contact or on laminin-coated coverslips	None	[77]
BM-MSC	Human Prostate Cancer - Du145	Spheroids	Forced Floating 96 well plate coated with Poly(2-hydroxyethyl methacrylate)	None	[117]
BM-MSC	Human Breast Cancer - MDA-MB-231 - MCF7	Scaffold	Polystyrene particles added to collagen I to form a Gel	None	[27]
BM-MSC	Human Breast Cancer - MDA-MB-231 - MCF7	Scaffold	Porous Scaffold composed nanocrystalline Hydroxyapatite (nHA) and Chitosan	None	[78]
WJ-MSC	Human Hepatic Cancer - HCCLM3	Contained Spheroids	Alginate Beads	Cisplatin	[118]
BM-MSC	Human Colorectal Cancer - HCT8 - DLD1 - Colo320DM - HT29 - HCT116 - T84	Spheroids	Forced Floating 96-well-plates coated with 0.7% SeaKem® GTG Agarose	None	[87]
WJ-MSC	Human Lung Cancer - AC-LCSC-229 and 223 - SCC-LCSC-136 and 36	Spheroids	Forced Floating Unspecified non-treated flasks to reduce cell adherence	None	[32]
BM-MSC	Human Lung Cancer - A549	Spheroids	Hanging drop 25000 per well in a tri culture method (ratios of 5:3:2 - A549 - Endothelial Lung Cells - Mesenchymal stem cells)	Paclitaxel and Gemcitabine	[89]
MM Marrow Derived MSCs	Human Multiple Myeloma (MM) Aspirates	Scaffold	Hydrogel composed by 0.5% PuraMatrix	IMiDs, Bortezomib, Carfilzomib, Doxorubicin, Dexamethasone, Melphalan	[82]
BM-MSC	Human Glioma - U-87 - U373	Spheroids embedded into a Scaffold	Spheroids embedded in Collagen I, Laminin or Matrigel	None	[92]

Figure legends

Figure 1. Human umbilical cord-derived mesenchymal stem cells interactions with human cholangiocarcinoma cell lines using a xenograft model and invasion assays. (A) MSCs capacity to migrate to the tumor site *in vivo;* (B) Increased growth of QBC939 cell line derived tumors; (C-E) increased resistance both in vitro and in vivo to the effects of the anticancer drug compound K, translated into higher rates of cell migration and cancer cell survival. Adapted from[119], with permission from Oncotarget under creative commons 3.0 license.

Figure 2. MSCs-cancer cell interaction is dependent on tissue of origin. MSCs can release several factors which can hinder or promote tumor progression at several levels. (red dots - tumor inhibition; blue arrows - tumor progression).

Figure 3. MSC migrate towards the tumor, with different mechanism playing major roles in diverse cancers (Glioblastoma -CCL2 and CCL25) (Hepatocarcinoma - CCL15 and CCL20). Migrated MSCs suffer a conversion process, releasing a myriad of factors that aid the tumor by rearranging the surrounding ECM, promoting tumor angiogenesis, proliferation, immune suppression and ultimately EMT and metastasis. [18,25,26,41,120].

Figure 4. Scaffold-based bone metastasis model. (A, B) Schematics of 3D *in vitro* platforms development and analysis; (C) SEM images of bone-mimetic scaffolds of chitosan and hydroxyapatite with or without previous MSCs culture derived modifications (D) MSCs co-culture with breast cancer cells (MCF-7 and MDA-MB-231) in bone-mimetic scaffold; (E) breast cancer cells evidenced a higher migratory capacity, with MDA-MB-231 evidencing the highest migration distance when co-cultured. This enhanced migratory capacity correlated with a higher expression of MTDH gene, which accompanied almost linearly increased co-culture

ratios of MSCs and breast cancer cells. Adapted from [78], with permission from Elsevier®.

Figure 5. Establishment of a hydrogel-based 3D multiple myeloma (MM) model. (A) Collection of aspirates from multiple myeloma (MM) patients in several disease stage; (B) MSCs populations characterization by flow-cytometry and classified as smoldering MM (SMM), newly diagnosed MM (ND), relapsed MM (REL) and relapsed/refractory MM (REF) patients; (C and D) After extensive characterization of retrieved MSCs and MM cells, cocultures where established both in 2D and 3D; (E) 3D to 2D comparison of co-cultured models showed significantly distinct cellular profiles, with 3D model presenting amongst other findings increased matrix deposition; Increased resistance of MM 2D and 3D models to (F) standard treatments, (G) immunomodulatory drugs and (H) proteasome inhibitors. Adapted from [82], with permission from Oncotarget under creative commons 3.0 license.

Figure 6. A549 and BM-MSCs 3D models co-culture performed in chitosan-hyaluronic acid coated wells. (A) Schematics of the biomaterial-based scaffold and workflow for assembly of 3D models; (B) Morphology of spheroids with diverse seeding ratios; (C) Fluorescence based analyzes of spheroid structure, 200 pm scale bar in panel; (D) Average diameter, total cell number of tumor spheroids, and cell ratio at 48h of co-culture. Adapted from [83] with permission from Elsevier®.

Figure 7. Breast-to-bone metastasis microfluidic chip-based model. (A) Schematics of the vascular heterotypic model with two channels for culture medium addition and injection of breast cancer cells and biochemical signals exchange with the collagen I hydrogel in the center channel EC - Endothelial cells (HUVECs), MSCs, OB - Obsteoblast differentiated cells (from MSCs lineage), CC- Cancer cells. All these cells were seeded in the hydrogel; (B)

Microvascular network formed in the hydrogel (HUVECs-green); (C to G) Evaluation of the established organotypic bone microenvironment, with Osteocalcin (red), Alkaline phosphatase (red), VE-Cadherin (red), ZO-1 (red) and a-smooth (red) biomarkers evaluation. Adapted from [121] with permission from Proceedings of the the National Academy of Sciences of the United States of America (PNAS).

Figure 8. Evaluation of cancer cell cannibalism towards MSCs. Upon cannibalism MDA-MB cells acquired an enriched molecular profile for pro-survival factors and tumor suppressor agents, as well as inflammatory mediators before entering a state of dormancy. (A) Scheme of co-culture method; (B) MSCs population decline visible in phase-contrast microscopy and flow cytometry; (C) MSCs cannibalism by MDA breast cancer cells; (D) MDA-MSCs in small aggregates as visualized through immunofluorescence imaging, prior to MSCs decline. Adapted from [101] with permission from Proceedings of the the National Academy of Sciences of the United States of America (PNAS).