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3D cell culture systems modeling tumor growth determinants in cancer target discovery ☆

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ABSTRACT

Phenotypic heterogeneity of cancer cells, cell biological context, heterotypic crosstalk and the microenvironment are key determinants of the multistep process of tumor development. They sign responsible, to a significant extent, for the limited response and resistance of cancer cells to molecular-targeted therapies. Better functional knowledge of the complex intra- and intercellular signaling circuits underlying communication between the different cell types populating a tumor tissue and of the systemic and local factors that shape the tumor microenvironment is therefore imperative. Sophisticated 3D multicellular tumor spheroid (MCTS) systems provide an emerging tool to model the phenotypic and cellular heterogeneity as well as microenvironmental aspects of in vivo tumor growth. In this review we discuss the cellular, chemical and physical factors contributing to zonation and cellular crosstalk within tumor masses. On this basis, we further describe 3D cell culture technologies for growth of MCTS as advanced tools for exploring molecular tumor growth determinants and facilitating drug discovery efforts. We conclude with a synopsis on technological aspects for on-line analysis and post-processing of 3D MCTS models.

Contents

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1. Introduction

Cancer is a complex disease dictated by diverse sets of oncogene gain of function and tumor suppressor gene loss of function mutations as well as local and systemic factors providing cues from the environment. Different combinations of cancer genes are mutated in various types of cancer. They drive the diverse phenotypes and characteristics of the malignant state in the context of a complex tumor tissue, where neoplastic cells reside together with many other cell types such as endothelial cells, fibroblasts and immune cells [1].

The importance of considering cell biological contexts and phenotypic heterogeneities during the multistep process of human tumor development is exemplified by the fact that despite significant progress in recent years resulting in the development of targeted anti-cancer drugs such as imatinib, trastuzumab, crizotinib and vemurafenib with improved response rates [2], the activity of these drugs is restrained. This is by and large due to cancer cell-intrinsic signaling cross talks and extrinsic interactions with other cell types as well as multiple components of the tumor microenvironment [3]. While the identification of recurrent mutations in diverse cancers is increasing with breath-taking speed through the application of next generation DNA sequencing [4] and a whole new arsenal of potential targets for the development of cancer therapeutics is emerging from these efforts, our knowledge about the complex intra- and intercellular signaling circuits occurring within and between the different cell types populating a tumor is still scarce. In addition, the interwoven regulatory networks generated by the diverse cell populations are further influenced by various physical and chemical parameters that form gradients, which altogether, contribute to the creation of different zones within the tumor and thereby to phenotypic heterogeneity [5]. Unraveling these connections is essential for realizing the goal of personalized genotype-directed cancer medicine.

The development and application of advanced three-dimensional (3D) cell culture models comprised of multiple distinct cell types provide not only an attractive approach to decode certain key aspects of intra- and intercellular signaling but also the contribution of non-genetic sources in the context of genetically and epigenetically diverse populations of cancer cells. 3D cell culture systems overcome many of the limitations of traditional two-dimensional (2D) monolayer cell culture systems by mimicking more closely the complex cellular heterogeneity and interactions and tumor microenvironmental conditions. They can also facilitate detailed mechanistic studies on the molecular and cellular reprogramming events that occur in different cell types during growth in a 3D culture format and offer opportunities for the discovery of new nodes in intra- and intercellular signaling networks and the validation of molecular targets in drug development.

In this review, we provide an overview of the various chemical, physical and biological factors in human tumors that require consideration when developing multicellular 3D cell culture model systems for translational cancer research and the applications of these systems in drug discovery.

2. Determinants of tumor growth in vivo

Human tumors encompass various cell types whose functions and fate are further differentially influenced by multiple chemical, physical and biological factors present in the microenvironment. Together, these constituents contribute to the development of different zones within a tumor mass [6–8]. Extensive communication occurs between these different cell types via heterotypic signaling and together with systemic and local factors, a heterogeneous tumor microenvironment is shaped that supports cancer cell proliferation, evolution, invasion and metastasis and contributes to therapy resistance (Fig. 1A).

2.1. Cellular components of the tumor microenvironment

Within a tumor, genetically altered, heterogeneous cancer cell populations [9] coexist with non-mutated progenitor cells (e.g. epithelial cells), cancer-associated fibroblasts (CAFs) [10], infiltrating immune cells [11] and endothelial cells. CAFs are considered to be activated or reprogrammed fibroblasts from various sources including normal fibroblasts and myofibroblasts [12]. Within the tumor stroma, such activated CAFs acquire a phenotype similar to activated fibroblasts during wound healing [13,14]. A prominent regulator of CAF-mediated cancer cell modulation is TGF-β. TGF-β is a secreted factor with multi-faceted functions during tumor evolution [15]. At early stages it can display potent tumor-suppressive functions [16], whereas at later stages it is a potent inducer of the epithelial to mesenchymal transition (EMT) important for the development of invasive and metastatic capabilities of cancer cells [17,18]. Fibroblasts play important roles in the deposition of extracellular matrix (ECM) and activated CAFs are known to be involved in tumor capsule formation as for example observed in hepatocellular carcinoma and renal cell carcinoma [19,20]. Fibroblasts have also been implicated in mediating release of growth factors and chemokines to influence cancer cell growth and to recruit endothelial cells and pericytes [10]. Additionally, they are hypothesized as potential source to fuel cancer cells with metabolites like fatty acids, pyruvate and lactate [21].

Co-culture models mimicking cancer–stromal cell interactions have been recently shown to present powerful tools to delineate cancer resistance mechanisms, as exemplified in the context of $BRAF^{V600E}$ mutant protein kinase signaling. Specifically, screening in seven $BRAF^{V600E}$ mutant melanoma cells co-cultured with 18 stromal cell lines allowed to uncover a novel mechanism of vemurafenib resistance mediated through secretion of hepatocyte growth factor (HGF) by six of the co-cultured stromal cells [22]. Other co-culturing experiments of mesenchymal stem cells (MSCs) together with breast cancer cells (BCCs) unveiled new mechanisms of paracrine signaling between MSCs and BCCs as promoters of metastasis, mediated by induced expression of the chemokine CXCL10 in MSCs and its cognate receptor CXCR3 in BCCs [23].

Since most genetically altered cancer cells are antigenic, a vast array of innate and adaptive immune cells infiltrate and contribute to the immunologic features of the tumor microenvironment and influence resident cells [24]. In this context, it is essential to understand the immunoregulatory processes of the tumor microenvironment as a whole for successful immunotherapeutic interventions like monoclonal antibody treatments in advanced melanoma with ipilimumab (anti-CTLA-4 antibody) [25].

Tumor growth and metastasis are fueled by a continuous supply of oxygen, nutrients and multiple growth promoting factors provided by the vasculature. Beyond a tumor volume of ~0.5–1.0 mm^3, diffusion is insufficient to provide all cells with enough oxygen resulting in tumor hypoxia. Hypoxia engages an adaptive program able to enhance oxygen delivery through the recruitment and activation of endothelial cells to vascularize the tumor and boost tumor growth [26,27]. This endothelial vascular system is a main source for non-cellular factors that influence the tumor microenvironment.

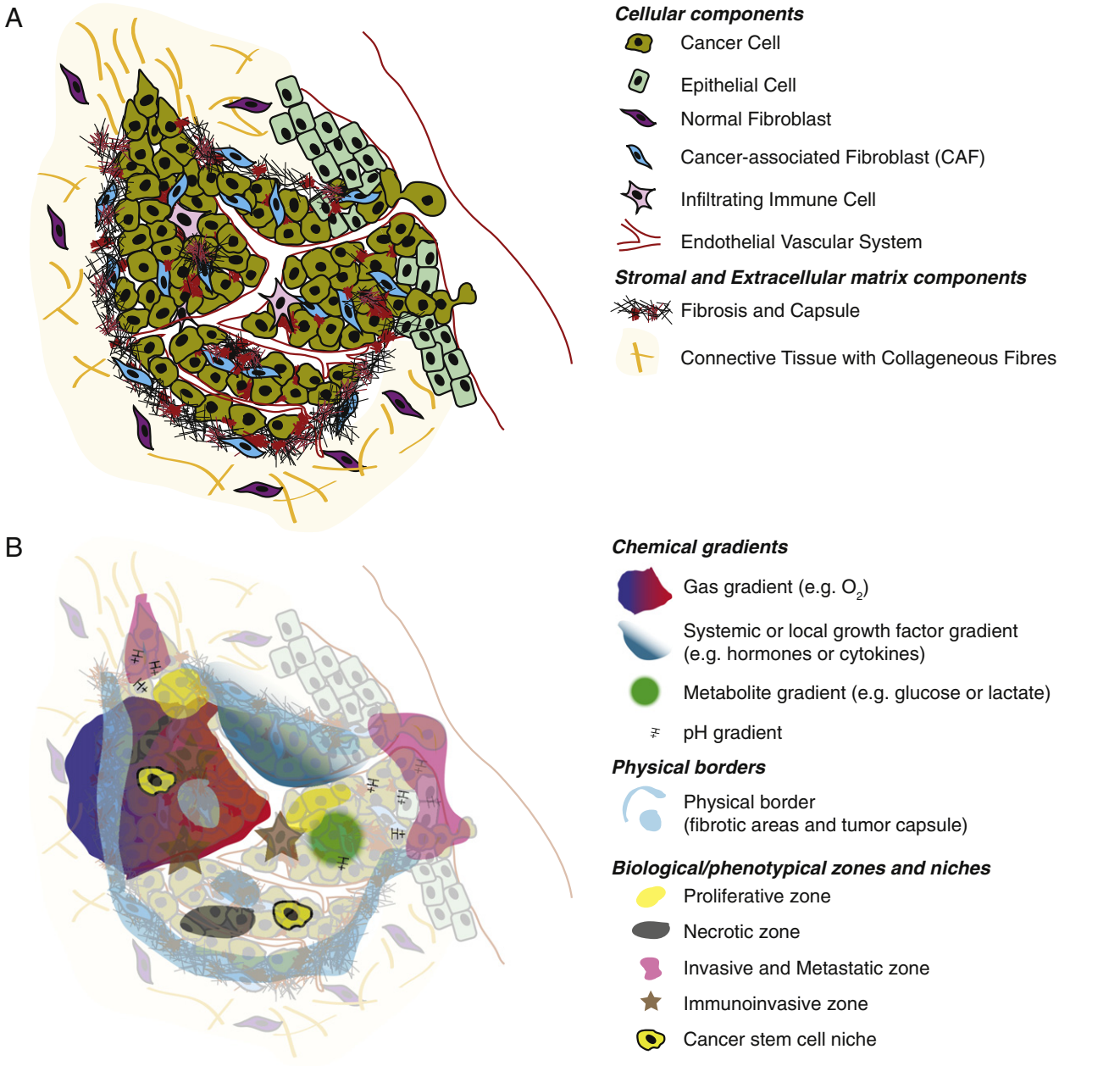

Fig. 1. The tumor microenvironment: (A) schematic illustrating cellular components (cancer cells and various stromal cells) coexisting in the tumor microenvironment and extracellular matrix components secreted by the cellular components of the tumor (see legend). The tumor is embedded in the connective tissue such as collagenous fibers. At the sites of cancer cell invasion, these collagenous fibers show perpendicular alignment. (B) The same scheme as in (A) except that chemical gradients, physical borders and biological/phenotypical zones and niches present in the tumor microenvironment are displayed (see legend).

Apart from promoting neovascularization, cancer cells reprogram their metabolism in a manner suitable for survival under hypoxia [28].

2.2. Chemical gradients and physical borders lead to biological/phenotypical heterogeneity in the tumor microenvironment

The non-cellular factors of the tumor microenvironment encompass chemical factors able to create gradients within tumors and physical factors displaying various forces and substrate properties. Accordingly, cells within the tumor microenvironment are differentially exposed to these chemical gradients and physical properties, which in turn, influence cancer cell properties as well as stromal cell functions. As a result, multiple biological/phenotypical zones form within the tumor microenvironment (Fig. 1B).

2.2.1. Chemical gradients: gases, systemic and local growth factors, metabolites and pH

A well-known elicitor of the previously mentioned zonation is the endothelial vascular system that supplies oxygen, nutrients and growth factors [29]. Supply of these factors for each individual cell in a tumor mass is influenced spatially through proximity to the vascular system and temporally by uncontrolled tumor cell proliferation as well as unstable or disturbed blood supply [30].

Spatial gradients arise as a result of diffusion and convection, with the latter defined as the collective movement of molecular aggregates in a fluid. They can also arise through local production and competitive consumption and turnover of the molecules by cells and proteins [31]. Gradients can be generated by gases (e.g. O_2), growth and hormonal factors and drugs used to treat cancer. These gradients can be further

modulated depending on proximity to the vascular system and diffusion limits [32].

Depending on type, gas gradients have very different properties and functions in the tumor environment. The free radical nitric oxide (NO) is highly unstable and has a half-life of 1–5 s in vivo [33], which results in steep local gradients from the sites of NO production in cells with high NO synthase activity. Depending on its concentration and the state of the tumor microenvironment, pro- and antitumorigenic functions have been attributed to NO [34]. In tumors as well as organs, interstitial mean oxygen decreases the farther the distance from blood vessels and a continuum ranging from light to severe hypoxia is observed. This is due to oxygen diffusion which is limited to approximately 130 μm of tissue thickness depending on the oxygen consumption rate of the tissue [35]. While hypoxia can be mild or even absent in very small tumors, larger tumors suffer from hypoxic regions due to an abnormal, often leaky vascularization, which results in an oxygen gradient. It is important to note that oxygenation in tumors is mostly not static but instead fluctuates temporally and regionally, which is also evident in heterogeneous local partial O_2 pressure measured in mouse tumor xenografts [36,37]. Fluctuations of hypoxia and reoxygenation differentially influence tumor cells and have other broad ranging implications for tumor progression, stress response, and signal transduction than permanent hypoxia [38]. Temporal changes in oxygen supply can be influenced by multiple factors including changes in the geometry of the microenvironment by de novo vascularization and vascular stasis [39], cell proliferation, changes in oxygen consumption rates of the surrounding microenvironment, or variations in red blood cell flux in the tumor vessels [40,41]. As a result, hypoxia leads to substantial reprogramming of cellular processes mainly mediated by hypoxia-inducible factor (HIF), a family of transcription factors mediating the primary adaptive response to changes in ambient oxygen concentrations [42–44]. Among the genes activated by HIF are also secreted factors acting in an autocrine, paracrine or endocrine fashion [45,46]. The secreted hypoxia-induced gene products promote profound changes in the tumor microenvironment. Among them are systemic hormones and local growth factors regulating hematopoiesis and angiogenesis such as erythropoietin (EPO) [47], vascular endothelial growth factor A (VEGF-A) [48] and platelet-derived growth factor B (PDGF-B) [49] as well as factors regulating the recruitment of inflammatory cells like stromal cell derived factor 1α (SDF-1α) [50]. Besides EPO, there are other systemic hormones influencing tumor growth which are delivered via the blood stream and build a gradient within the tumor microenvironment, such as estrogens, which strongly influence breast cancer development [51] and testosterones that impact on prostate carcinoma progression [52].

Cancer cells are strictly dependent on continuous nutrient supply to support proliferation and survival. Metabolic reprogramming is a consequence and necessity to fulfill this high demand. Genetically, this can be achieved directly by deregulation of metabolic enzymes, for example through mutations of isocitrate dehydrogenase 1 and 2 genes (*IDH1* and *IDH2*), which have been described in the majority of gliomas [53] and by mutations in succinate dehydrogenase complex subunit D (*SDHD*), that were found in paragangliomas [54]; or indirectly, through reprogramming by oncogenic signaling for example as shown for oncogenic mutations in *MYC*, *SRC* or *RAS* genes [55–57]. Furthermore, changes in cellular metabolism are also tightly connected to changes in oxygen concentrations. One of the most prominent intrinsic cellular changes induced by HIF is a profound alteration in expression levels of metabolic genes leading to a shift from an oxidative to a glycolytic form of metabolism. This is mediated, at least in part, by HIF-dependent upregulation of glucose transporters [58] and glycolytic enzymes to ensure a higher flux through glycolysis and upregulation of pyruvate dehydrogenase kinase 1 (PDK1) that inhibits the function of pyruvate dehydrogenase thereby limits pyruvate from entry into the tricarboxylic acid (TCA) cycle resulting in increased lactate production [59]. This reprogramming of metabolism together with an increase in glucose consumption and lactate production results in the generation of chemical metabolic gradients as well as pH deregulation in conjunction with increased carbonic anhydrase IX (CAIX) expression [60]. Most cancer cells take advantage of this metabolic, a phenomenon called the "Warburg effect". On first sight a paradox, accelerated flux of glucose and increased glutamine uptake provide both ATP, NADPH and carbon skeletons for macromolecular synthesis such as nucleotides and backbones for lipid synthesis. Thus, cancer cells are reprogrammed with respect to their metabolism in a manner that sustains the uptake of large amounts of nutrients such as glucose, glutamine, essential amino acid and in certain contexts even fatty acids to fuel anabolic pathways supporting their relentless growth and division [61].

Gradients of metabolites are mainly dictated by turnover/consumption rates of cells and external supply. As mentioned before, cancer cells are highly dependent on nutrients including glucose, especially those located in hypoxic environments. Sonveaux et al. have proposed a concept of metabolic symbiosis as a rationale for sufficient glucose delivery to the hypoxic niche [62]. It is shown that certain types of cancer cells can utilize lactate under aerobic conditions through uptake of this metabolite from the environment via monocarboxylate transporter (MCT)1. Lactate is then converted to pyruvate for the use in oxidative phosphorylation by lactate dehydrogenases. This "metabolic symbiosis" is the result of an interplay of oxygen and pH gradients, both resulting in spatially different reprogramming of cancer cells to ensure tumor homeostasis via metabolic compartmentalization, as observed, for example, in head and neck cancers [63]. Interestingly, this homeostasis could be disturbed by pharmacologically blocking MCT1, forcing the cells to utilize glucose instead of lactate thereby starving hypoxic glucose-consuming cells [62]. Due to the fermentative metabolism and resulting excessive lactate production, cellular pH balance is essential [64,65]. Besides the MCT4-mediated active lactate export out of hypoxic cells to counter-balance acidification of the intracellular milieu, carbonic anhydrases also contribute to maintain a balanced pH [66]. Together with poor perfusion, these processes can lead to acidification of the tumor microenvironment and the formation of extracellular pH gradients that are found in solid tumors (Fig. 1B) [66].

2.2.2. Physical borders

Physical borders are defined here as the gradual change of a physical property such as material stiffness, porosity, and topology mostly due to ECM deposition. Such functional borders are found in most benign solid tumors, either within the tumor where fibrotic areas result from extensive ECM deposition or surrounding the tumor as a tumor capsule (Fig. 1A). A tumor capsule is composed of connective tissue and ECM components (mostly secreted by activated myofibroblasts), which can entirely engulf and constrain the tumor. While cancer cells can utilize various mechanisms (e.g. EMT, budding, and circulating tumor cells [67–69]) and routes (blood, lymph, ECM [70]) to metastasize, an undisrupted capsule also hampers the tumor cells' invasiveness and metastasis and is often indicative of good prognosis as for example observed in prostate cancer [71]. However, such an undisrupted capsule can also be a barrier for drugs in pancreatic ductal adenocarcinoma [72] or immune cells [73]. When disrupted and the desmoplastic collagen aligns perpendicular to the tumor, invasion and metastasis of cancer cells are then facilitated (Fig. 1A) [74]. In addition to the invasive routes via the connective tissue [74,75] as well as through lymphatic vessels [76], tumor invading blood vessels offer another route for metastasizing cells [77]. Clearly, the elasticity and contractility of the surrounding tissue contribute significantly to the physical characteristics and density of the capsule [78].

2.2.3. Biological/phenotypical zones in the tumor microenvironment

Both genetic heterogeneities of cancer cells and microenvironmental cellular, chemical and physical compositions contribute to biological/phenotypical zones within a tumor mass. Such zones can be created through different metabolic strategies employed by the various cell types of a tumor. In this regard, metastatic niches often correspond to

areas of highest acidity, thus lowest extracellular pH [79]. Secretion of chemokines by the microenvironment and expression of adhesive molecules on the vascular endothelium that attract immune cells result in invasive zones. Intriguingly, T cells infiltrating tumors have a significant prognostic value [80–82]. Moreover, not all cancer cells have the capacity of de novo tumor formation. Rather, it is only a small fraction of cancer cells often referred to as cancer stem cells (CSCs), which reside in a CSC niche, that display such a capacity [83] (Fig. 1B). This niche is highly influenced by many tumor microenvironmental factors, like immune cells, vascularity, soluble factors and ECM, which contribute to stemness, proliferation and apoptosis resistance of the CSC [84]. Furthermore, it is important to recognize that the multitude of pathophysiological factors shaping different phenotypical/biological zones and CSC niches in the tumor microenvironment is not static but can fluctuate in space and time.

The resulting phenotypical/biological zonation has a profound impact on tumor responses to therapy. Therapy resistance (e.g. radiotherapy or chemotherapeutic agents such as cisplatin or doxorubicin) is often tightly associated with hypoxia [85–88]. Furthermore, altered metabolism influences sensitivities to therapeutic compounds [65,89]. Consequently only certain tumor zones may respond to therapy but not others. Additionally, like chemical factors (Section 2.2.1), many drugs are administered via the bloodstream and cannot reach the collectivity of target cells in a vasculature-deprived environment. Even when they reach the tumor, they likely present themselves in the form of gradients. Therefore, tracking therapeutic responses in models that better reflect an in vivo situation, where for example, constant oxygen and nutrient restrictions are dominating and not all cells are rather well-nourished and oxygenated as it occurs in a 2D cell monolayer, will be vital for understanding how tumor cells embedded in a heterogeneous microenvironment respond to therapy and what restricts therapeutic responses.

3. 3D tumor spheroids as models for in vivo tumor growth determinants

Delineating the various reciprocal interactions between the different cell types in a tumor and elucidating the underlying principles of microenvironmental influences will prove critical to the understanding of cancer pathogenesis and the development of novel, effective therapies. 3D tumor spheroid models can close the gap between 2D tissue culture models and in vivo whole-animal systems to study tumor biology and drug responses. Compelling evidence from studies over the last two decades has shown that growing cells in 3D, using various methodologies, can resemble many physiological in vivo aspects significantly better than cells grown in 2D monolayers [90–92]. Nowadays, these models have been developed further to allow drug and genetic screening in high-throughput formats [93–95]. Therefore, 3D models and technologies have become an invaluable tool for accelerating translational research and drug discovery. Importantly, cells adapted for growth in 2D do not necessarily grow also as 3D spheroids. However, if they do so, their growth in 3D is suitable to model at least some of the above-mentioned chemical gradients and physical borders (Section 2.2) leading to biological/phenotypical zonation within the spheroid.

Signaling pathways, for example, can show different activities in 3D versus 2D culture models. Breast cancer cells form HER2 (ERBB2) homodimers in HER2-positive breast cancer patients and 3D cultures, resulting in effective growth inhibition by trastuzumab. In contrast, in 2D cultures, HER2/HER3 heterodimerization is preferentially found [90]. Moreover, HER2 homodimers activate in 3D mitogen-activated protein kinase (MAPK) signaling, while in 2D, HER2/HER3 heterodimers favor the activation of the phosphoinositide 3-kinase (PI3K) signaling pathway [90]. Similarly, increased IL-6 secretion and activation of STAT3 are observed in a Ras-transformed subculture of mammary epithelial cells when cultured in 3D, a phenotype also seen in xenografts and human tumors, but not in standard 2D cultures [96]. Enhanced production of IL-6 has been linked to drug resistance [97,98], and consequently, STAT3 activation is required for invasion and migration [96]. Therefore, 3D models provide a layer of complexity important for delineating cancer cell signaling mechanisms as well as drug responses.

Multiple levels of complexity can be modeled in 3D cultures ranging from simpler models of monocultures of cancer cell lines in liquid-based environments incorporating oxygen/nutrient gradients and cell–cell-interactions to more advanced models that include co-cultures with other cell types such as endothelial and immune cells. Growth of cells in

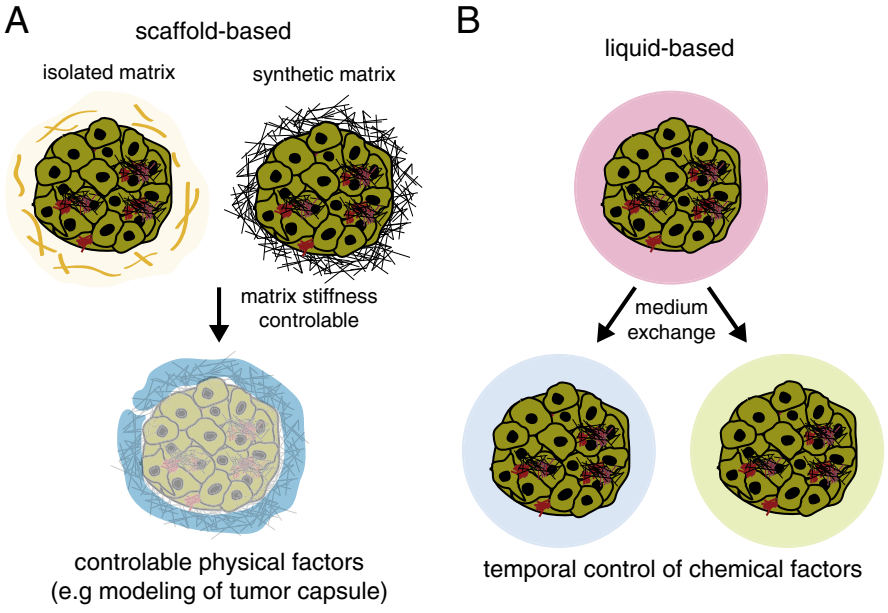

Fig. 2. Scaffold and liquid-based 3D tumor microspheroid models: schematic illustration of the two main 3D tumor microspheroid models and their chief distinguishing features. Tumor spheroids are schematically illustrated as monocultures of cancer cells with areas of secreted extracellular matrix. (A) Scaffold-based models either with a natural-isolated matrix (e.g. Matrigel, left) or synthetic matrix (bottom) controlling matrix stiffness and influencing physical factors. (B) Liquid-based models allow medium exchange to temporally control chemical factors.

scaffold-based environments allows incorporating external physical properties. These models will be presented and discussed in the context of the afore-mentioned "determinants for tumor growth in vivo" (Section 2).

3.1. 3D tumor spheroid models for target identification, validation and lead compound discovery

Various systems exist to grow 3D spheroids with different properties. Unfortunately there are no guidelines as to best grow various cells in mono- or co-cultures in 3D to mimic specific aspects of the in vivo situation. Here we consider two main categories of 3D culture systems: scaffold-based and non-adherent, liquid-based (Fig. 2).

Scaffold-based systems provide a platform where cells are grown in a semi-solid matrix that allows the study of influences of mainly external physical factors on microspheroid growth and signaling [99]. Scaffolds can consist of natural matrix isolates that are less defined. Such natural ECM isolates can be derived from decellularized tissues and organs [100–102], which have been widely explored in regenerative medicine, or from cultured cells like fibroblasts, chondrocytes and MSCs [103,104]. One of the most used and commercially available natural matrix isolates is secreted by Engelbreth–Holm–Swarm (EHS) mouse sarcoma cells [105] (e.g. Matrigel® or Cultrex®). Other natural matrices are hydrogels such as gelatin, collagen, laminin, or alginate gels [106–108], or derivatives and combinations of these. Alternatively, synthetic scaffolds are available as well-defined synthetic polymers including poly(ε-caprolactone) [PCL] or polyethylene glycol [PEG] [99,109,110]. Most of these scaffold-based systems allow to control spheroid size by adjusting pore-size and density [111]. The use of scaffolds has improved the diversity of 3D culture models by incorporating additional factors such as stiffness and porosity of the matrix to closer mimic certain tumor microenvironmental aspects. These physical factors can only be incorporated using scaffolds and have been shown to alter gene expression and cell signaling [112]. An advantage of specialized scaffolds is that they can be customized to fit the experimental purpose and the needs of certain cancer cell types [92,113]. For example, the invasion of gliomas through neural tissue occurs along elongated anatomic structures, which could be mimicked in vitro in 3D by the use of poly-e-caprolactone nanofiber scaffolds, while liquid-based methods could not provide such a specialized support [114]. However, the choice to customize scaffolds also leads to the need for careful characterization and validation of the chosen system, and it appears that only few of these methods are sufficiently characterized to accurately resemble the tumor architecture and environment [91]. A disadvantage for spheroids grown with the support of a matrix is the difficulty to temporally modify the extracellular environment to administer chemical components like growth factors, metabolites and drugs. In this context liquid-based cultures are advantageous, where medium exchange allows these temporal modifications. However, some artificial cell-matrices exist where the experimenter can actively modify matrix properties over time such as the biophysical properties (e.g. stiffness) or biochemical properties through coupling of growth factors. Photoclick chemistry in PEG hydrogels using reactive thiolene groups to couple various ligands in the presence of cells allowed to modify matrix properties in real-time [115]. While scaffold-based systems are crucial for studying the influence of extracellular biophysical properties such as modeling of stroma or a tumor capsule (Fig. 2A), analysis often requires more creative and tedious solutions depending on the properties of the matrix. Once the spheroids are embedded in the matrix, post-processing and isolation of single microspheroids can present a challenge.

Most non-adherent, liquid-based systems allow the exchange of medium to a certain degree, although careful handling in most systems is necessary due to lack of adherence of the microspheroids (Fig. 2B). Four main liquid-based systems are available: (i) the hanging-drop technology, where cells are grown in a drop to form one single spheroid; (ii) the growth on non-adherent surfaces such as specially (un)coated plates (ultra-low attachment) or micropatterned plates that prevent attachment; (iii) microcarrier systems, where cells/spheroids aggregate on coated carriers and (iv) rotation-based culture methods such as spinner flasks or rotary cell culture system (RCCS), which facilitate microspheroid formation by collision. All of these methods have their advantages and limitations. The advantage of both hanging drops and non-adherent surfaces (U-shaped wells) is that only one single spheroid is formed of highly reproducible size [94,95]. While medium exchange using the hanging-drop method is challenging (unless spheroids have been transferred to observation plates), spheroids can be observed and modified at all times using non-adherent surfaces. If flat-bottom micro-patterned surface plates are used, multiple spheroids/aggregates of various sizes are formed, which might add experimental noise. Microcarrier systems allow the exchange of medium if magnetic carriers are used, while RCCS and spinner cultures allow the growth of microspheroids in high quantities. A disadvantage of rotation-based culture is that the size of 3D spheroids is often variable and the maximum size is limited by the stirring/rotation speed.

Finally, organotypic systems most closely resemble an organ as ex vivo model. Such systems exist for several organs including the liver, kidney and lung [116–118]. Organotypic systems can be derived from organ slices or differentiated 3D stem cell cultures [119,120]. The equivalent for modeling tumor growth determinants in cancer target discovery would be direct isolates from tumors grown as 3D cultures, tumor slices or genetically modified 3D stem cell cultures that differentiate as tumor-like structures.

A summary of selected 3D culture methods most suitable for cancer target discovery applying screening technology is shown in Table 1, and multiple reviews exist, discussing in more details the properties of liquid-based and/or scaffold-based 3D culture systems [99,121]. Since this review focuses on 3D growth systems that are compatible with high-throughput screening modalities for drug target identification and validation, microfluidic and chip-based methods will not be discussed, even though these systems allow environmental adjustments in a very controlled way, being of great value when exposing single spheroids to chemical factors in time-dependent manners followed by high-content single microspheroid on-line analysis [122].

3.2. 3D tumor microspheroids can model multiple determinants of tumor growth in vivo

Various models of 3D tumor spheroids allow reconstructing specific aspects of in vivo tumor growth within certain limitations. In the following sections models mimicking cellular heterogeneity, features of the tumor microenvironments, chemical gradients and intratumor zones will be discussed.

3.2.1. Multi-cell-type 3D tumor microspheroids to model cellular heterogeneity

As introduced in Section 2, a solid tumor is not only composed of mutated cancer cells, but is also an entity of various cell types. Although a 3D system with cancer cell lines cannot recapitulate the genetic heterogeneity found in tumors, modeling cellular heterogeneity can be achieved to a certain degree with heterotypic multi-cellular models [94,123]. All 3D models, as introduced in Section 3.1 are suitable for performing co-culturing studies, particularly when different cell-types are initially mixed prior to seeding for 3D growth. Furthermore, most liquid-based models allow also sequential addition of the various cell-types, which results in layered spheroids to study microspheroid invasion, e.g. by immune cells. Examples of 3D co-culture models depicted schematically or as 3D microspheroids grown using the hanging drop technology and processed by immunohistochemical staining are shown in Fig. 3. Exemplifying a phenotype elicited in the cancer cells only after co-culturing with fibroblasts is a co-culture of HepG2 cells

Table 1
Selected 3D culture methods suitable for cancer target discovery applying screening technology.

	Scaffold-based		Liquid-based				
	Synthetic polymer matrix	Natural polymer matrix	Hanging drop technology	Pellet culture in U-bottom ultra-low attachment plates	Micro-molded surfaces	Micro-patterned plastic surface plates	Spinner flask or rotary cell culture systems (RCCSs)
Size control	+/0 Multiple non-uniform MCTS	+/0 Multiple non-uniform MCTS	++	++	+++ Max. size defined by pore size	0 Multiple non-uniform MCTS	0 Multiple non-uniform MCTS
Heterotypic cell ratio control	+/0	+/0	+++	+++	+++	0	0
Chemical environment control							
Initial	+++	+	+++	+++	+++	+++	+++
Temporal	+	+/0	++	+++	+++	+++	+++
Comment	Initial polymer modifications possible as well as temporal (limited)	Batch dependencies might hamper reproducibility	Temporal control needs transfer to culture plate				
Physical environment control							
Initial	+++	++	0	+/0	0	0	0
Temporal	+	0	0	0	0	0	0
Comment	Initial polymer modifications possible as well as temporal (limited)		Addition of matrix possible at low amounts	Addition of matrix possible at low amounts			
Spheroid yield	Medium/low	Medium/low	Low	Low	Low	Low	High
Comment	Multiple per well	Multiple per well	1 per well	1 per well	1 per well	Multiple per well	Multiple per vessel
Labor intensity	+++	+++	++	+/0	++	+/0	0
Comment		For natural non-commercially available isolates even more labor intense					
Analysis							
On-line	+/0	+/0	+	++	+	+++	0
Post-processing	+/0	+/0	+++	+++	+++	+++	+++
Comment	On-line imaging limited; post-processing limited due to matrix embedding	On-line imaging limited; post-processing limited to low amounts; post-processing limited due to matrix embedding	On-line analysis suitable after transfer to observation plates	Post-processing limited to low amounts	Post-processing limited to low amounts	Post-processing limited to low amounts	Post-processing limited to bulk amounts
Screening compatibility							
Arrayed	++	++	+++	+++	+++	++	0
Pooled	+/0	+/0	0	0	0	+/0	+++
Comment	Pooled screening limited by low yield, bigger labware needed	Pooled screening limited by low yield, bigger labware needed				Pooled screening limited by low yield, bigger labware needed	

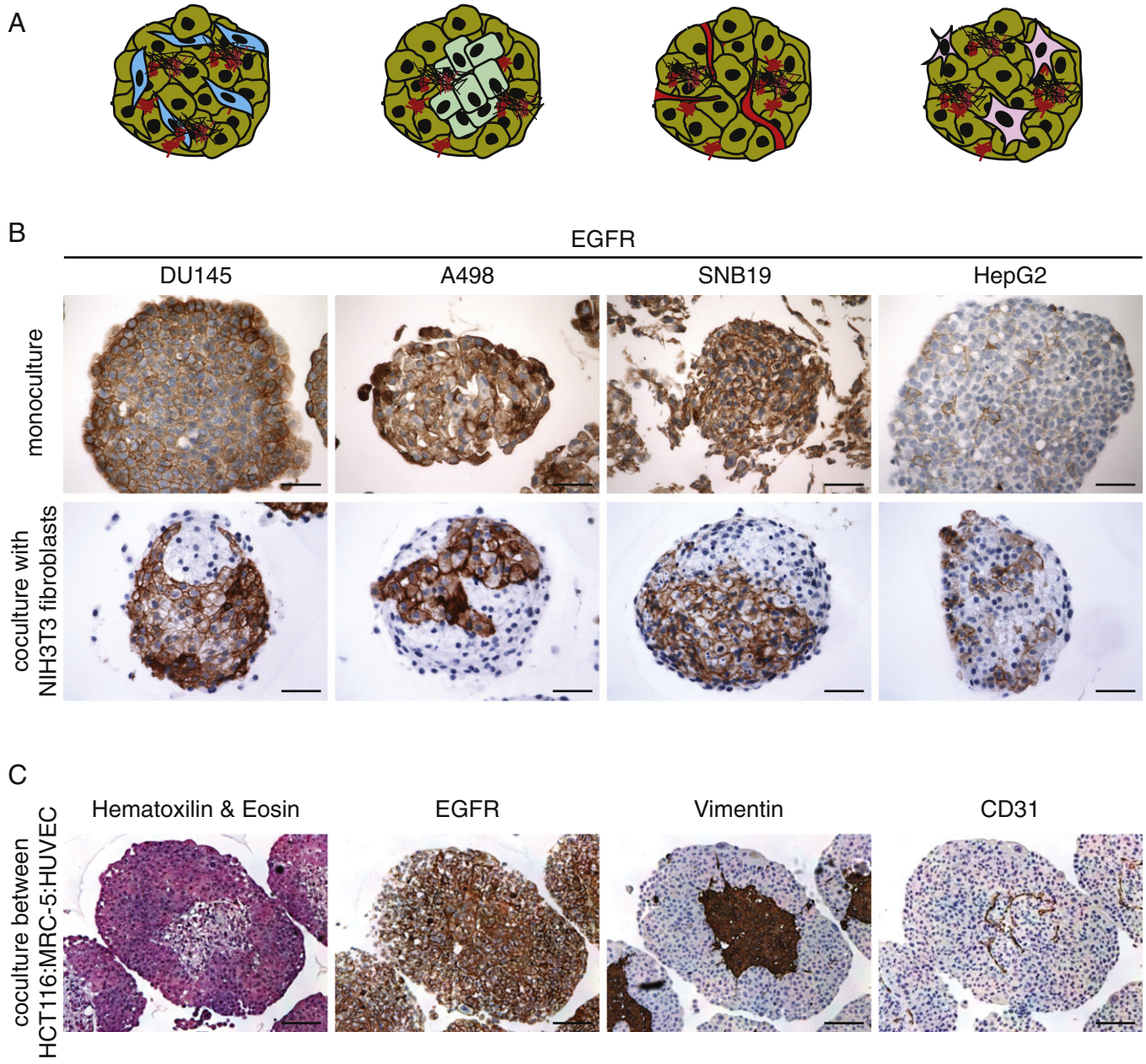

Fig. 3. Examples of various cancer cell:stroma 3D co-culture models: (A) schematic illustration of multi-cell-type 3D tumor microspheroid co-culture models using hanging drop technology with combinations of cancer cells and stromal cells. (B) Immunohistological stainings for epidermal growth factor receptor (EGFR) of prostate cancer (DU145), kidney carcinoma (A498), glioblastoma (SNB19) or hepatocellular carcinoma (HepG2) tumor microspheroids grown as monocultures or co-cultures with murine NIH3T3 fibroblasts. Scale bar = 50 μm. (C) Hematoxylin & eosin stainings and immunohistochemical analysis of EGFR, vimentin and CD31 of consecutive sections of tumor microtissues generated from colorectal cancer cells (HCT116) cocultured with human MRC-5 fibroblasts and human umbilical vein endothelial cells (HUVECs). Scale bar = 100 μm.

with NIH3T3 fibroblasts leading to an upregulation of the epidermal growth factor receptor (EGFR) on the cancer cell surface compared to the single cell type cultures (Fig. 3B). A further increase in the complexity of a 3D tumor spheroid model can be achieved by implementing endothelial cells as demonstrated in Fig. 3C. In this example, the colorectal cancer cell line HCT116 is grown together with MRC-5 human fibroblasts and human umbilical vein endothelial cells (HUVECs). The endothelial cells are nesting in the connective tissue compartment generating capillary-like structures. Even though it is questionable if such capillary-like structures are functional and as such are able to facilitate the exchange of gases and/or the delivery of chemical factors from the surrounding medium, such a model could be useful to study the induction of angiogenesis and recruitment of endothelial cells into hypoxic regions as found in human tumors.

3.2.2. Chemical gradients elicit the generation of biological/phenotypical zones in 3D microspheroids

Spheroid formation proceeds through multiple stages, starting from initial seeding of single cells to aggregation. Due to the 3D shape of the growing microspheroids, internal chemical gradients start building up. This leads to a typical zonation from the surface to the core as observed in growing tumors in vivo [30]. This is best illustrated through analysis of a developing hypoxia gradient [124]. Due to the diffusion limit of oxygen, spheroids become hypoxic in the center, which can then lead to

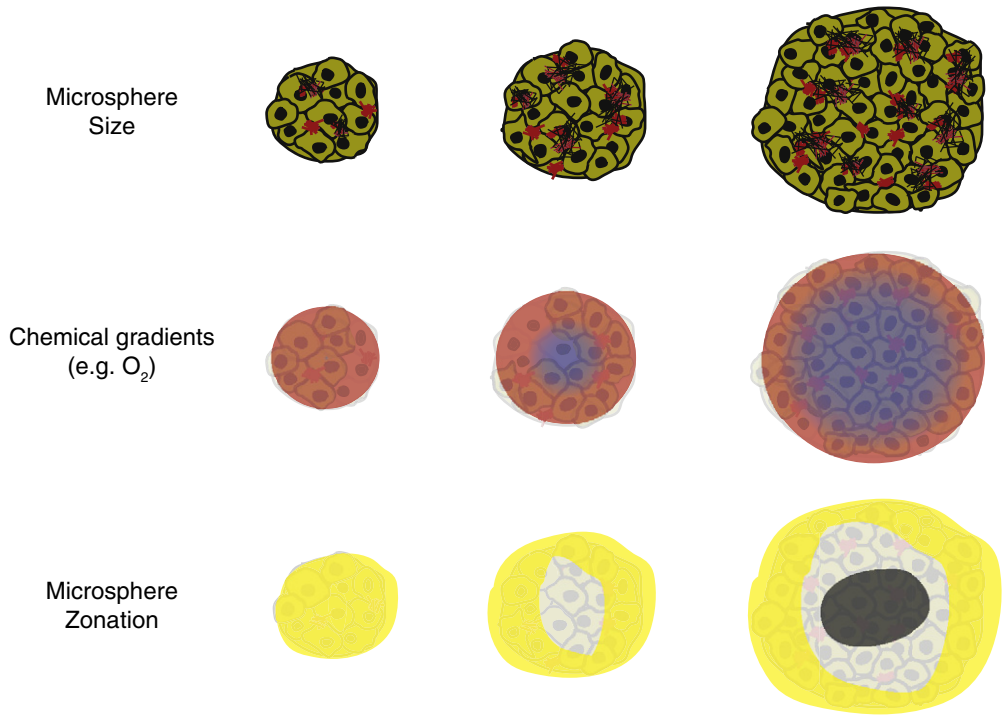

Fig. 4. 3D tumor microspheroid size, chemical gradients and biological/phenotypical zonation: schematic of 3D tumor microspheroid monoculture models illustrating how microspheroid growth (top) generates gradients (middle; e.g. O_2) followed by typical zonation (bottom; yellow: proliferative zone, black: necrotic zone).

necrosis in larger diameter spheroids [125,126]. Initial seeding and aggregation followed by formation of a small microspheroid with proliferating and mostly normoxic cells do not show striking zonation since chemical factors diffuse freely. Further growth to medium-sized spheroids with diameters of app. 150–300 μm starts to show a typical zonation from the surface to the core with a proliferative zone at the surface, normoxic quiescent zones in the middle and a hypoxic zone in the core [127]. The core of the hypoxic zone is in large spheroids anoxic and can develop, upon further growth, a necrotic zone (Fig. 4) [128]. Although regulation of microspheroid size seems to be trivial, generating the optimal size to study determinants of tumor biology needs careful assessment of the growth properties and microspheroid characteristics, since the tightness of cellular interactions within the spheroid can additionally limit diffusion of chemical factors and is cell-type dependent [95]. A small tight microspheroid of 200 μm may already be hypoxic, while a loosely aggregated spheroid of 400 or 500 μm may not [91]. Oxygen tension measurements in multicellular spheroids with O_2-sensitive microelectrodes show a gradual decrease towards the center as a consequence of diffusion and consumption rates [124]. In addition, measurements of respiratory activity in microspheroids from human MCF-7 breast cancer cells using hanging-drop and micro-molded surface technologies demonstrated that the respiratory activity of spheroids increased with spheroid volume until the radius reached 200–250 μm, concomitant with the emergence of a necrotic core. However, total respiratory activity was dependent on the 3D culture method used [129].

Comparative gene expression studies in melanoma cells grown in 2D and 3D confirmed the emergence of a hypoxic core through upregulation of multiple HIF target genes [130]. Similar observations were made in two studies using squamous cell carcinoma cells. In one study inducers of tumor vasculature in 3D spheroids emerge in an integrin-dependent manner [131] while in another study a reprogramming of HIF-regulated metabolic genes was demonstrated [132]. The latter could in principle allow 'metabolic symbiosis' as proposed by Sonveaux et al. [62]. Furthermore, multiple studies show strong induction of secreted factors such as interleukins, chemokines and growth factors that could potentially contribute to cross talk between various zones within the spheroid [133–135].

Tight control of spheroid size is therefore necessary to obtain reproducible results, and depending on the scientific question, actual microspheroid diameters may vary. For studies of tumor initiation, smaller well-oxygenated spheroids with an optimal diameter of approximately 200 μm are desirable, whereas for studies relating to tumor expansion, larger microspheroids are preferred in order to model the hypoxic and necrotic areas observed in poorly vascularized tumors. To which extent an appropriate combination of cells, chemical factors and gradients allow for modeling a CSC niche in spheroids remains still unexplored. The geometric properties of 3D microspheroids also permit to study simultaneously intra- and intercellular crosstalk of cells in different zones [136]. The application of appropriate sensors that interrogate activities of specific signaling pathways engaged by various factors has to be employed to distinguish the responses in various cell-types as well as the various cellular phenotypes generated through zonation in the 3D tumor microspheroid.

3.3. Methods and sensors to study tumor mechanisms with 3D microspheroids for target and drug discovery

When scaling-up to the third dimension, live cell imaging represents a powerful tool to interrogate cell function and behavior. Genetically encoded sensors are mainly useful when working with cell lines whereas chemical sensors are easier to employ as they can freely penetrate in many cases the microspheroid. An important aspect to consider when imaging 3D spheroids is the limitation of spheroid penetration, Z-axis resolution, image depth and light scattering. Therefore, most studies in 3D microspheroids are either done at lower resolution, where the

microspheroid as a whole is observed or using microscopy with a better Z-resolution for single cell observations, e.g. by multiphoton microscopy. The latter approach is very time consuming and would not allow for high-throughput. Additional post-processing like harsh treatment with *Scale* fixative reagent [137] enhances detection depth of 3D spheroids (application note, PerkinElmer) and still allows for higher throughput imaging with optical instrumentation. Post-processing of microspheroids by embedding and sectioning delivers very high detection depth but is also very time-consuming. However, both afore-mentioned methods are not compatible with live-cell imaging as two-photon microscopy is.

Molecular analysis of multi-cell-type 3D microspheroids requires markers that enable sorting of the different cell populations to deconvolve the relative contribution of each cell type to cell function in the microspheroid [138]. The most simple way to distinguish e.g. different cell types, is by using colored dyes, such as CellTracker™, that are taken up by cells prior to spheroid formation, but care has to be taken as the signal diminishes after each cell doubling. Alternative markers are represented by stably-expressed fluorescent proteins or genetic barcodes. The most simple genetic sensors successfully employed in co-culture experiments using multi-cell type 3D microspheroids are the stable expression of different fluorescent reporter proteins [94]. This allows monitoring of the growth properties of each cell type in situ.

3D microspheroids are a useful tool to study the hypoxia sensing and signaling through geometry, as it happens in vivo [139]. As opposed to experiments using hypoxia chambers where cells are grown in 2D, hypoxia occurs gradually and slowly over time and simultaneously to microspheroid growth, and not acutely through transfer to a hypoxic environment. This permits an assessment of the impact of spatial and temporal changes in oxygen levels on cancer cell proliferation [140]. To visualize hypoxia, a genetically encoded sensor based on integration of a luciferase reporter cassette driven by a hypoxia-response element (HRE) has been employed in Panc-1 spheroids to discover HIF-1α and sonic hedgehog (SHH) driven activation of the hedgehog signaling pathway [141]. A similar sensor using HRE-driven DsRed expression was used to study how culture geometry in 3D controls hypoxia-dependent VEGF secretion [142]. An alternative to the stable expression of a hypoxia reporter is the application of chemical sensors such as HypoxiSense 680 (PerkinElmer) [143], a read-out for CAIX activity, or Hypoxia Probe LOX-1 (Scivax Inc.), an iridium-complex-based phosphorescence probe that is quenched by molecular oxygen under ambient oxygen conditions [144]. Application of these chemical probes allows to visualize hypoxia in real-time in 3D microspheroids. Pimonidazole has also been used to detect hypoxic areas in patients, rodents and 3D spheroids, but requires post-processing of the spheroid using immunohistochemistry [145].

To study metabolic changes elicited through either oxygen or metabolite gradients, innumerable glucose sensors exist with a wide range of applicability allowing the measurement of glucose concentration as well as consumption and metabolic rates [146]. Measurements of total metabolic rates of 3D microspheroids, using fiber-optics-based sensors or with single-cell genetic sensors based on glucose-binding protein (GBP) fused to fluorescent proteins, allow assessing metabolic rates of 3D microspheroids. Also multiple methods and fluorescent small-molecule pH sensors exist with different characteristics and applicability for measuring extracellular and intracellular pH [147,148]. Production of lactate in cancer cells is a main cause for changes in pH. Interestingly, a genetically encoded lactate sensor to quantitatively detect lactate in single cells based on Föraster resonance energy transfer (FRET) has been applied in 2D but not yet in 3D culture systems [149]. The application of FRET in 3D requires however complex instrumentation [150].

The study of crosstalk of cells in 3D microspheroids requires a careful dissection of the various cell types within the entire cell population. Single-cell metabolic profiling from layers of HCT116 tumor microspheroids to study glycolipid metabolism provides an interesting example. After incubation of spheroids with fluorescently labeled glycosphingolipid substrates, sequential removal of TMT layers with serial trypsinization allowed the isolation of single cells from specific layers of the spheroid. Depending on localization within the microspheroid, the specific layers of cells displayed clear differences in glycosphingolipid metabolism as a function of metabolite accumulation [151].

The application of spheroid technologies to anti-cancer drug screening regimes and other high-throughput screening modalities is adding additional challenges and for many of these technologies robotic handling systems are required to mainly manage the labor-intense spheroid formation, spheroid transfer steps and medium exchanges (Table 1). It should be however said that screening applications, which are achievable without expensive robotic equipment manually could be employed in many research labs. For example pooled growth of spheroids in RCCS and spinner cultures in high quantities presents an excellent model for genetic screening with pooled RNAi-based technologies and their subsequent deconvolution using genetic barcodes [152]. For arrayed screening technologies plastic-ware in micro-plate format for e.g. hanging-drop technology or ultra-low attachment plates is available and is applicable for medium-throughput. A higher throughput can be achieved with a recently developed micro-scaffold array chip technology using polymethylmethacrylate (PMMA) chips, that allow for fast medium exchange and arrayed drug administration [153]. In general, multiple sensors and technologies are available to study and screen for tumor characteristics in 3D microspheroids. In each case, however, growth conditions need to be adapted and appropriate processing methods and instrumentation be applied to get maximum resolution and to minimize noise.

4. Conclusions

Although the creation of multi-cell type 3D microspheroids is challenging, this system offers many advantages over 2D tissue culture models. This is particularly well exemplified by the different chemical and physical gradients and the consequent phenotypic heterogeneity these gradients produce during growth of cells as 3D microspheroids. As presented in Section 3.1 and Table 1, there is a multitude of different models for growing tumor cells in 3D. Each model has its own strengths and weaknesses with respect to mimicking the various aspects of relentless tumor growth. Since dependencies on different tumor growth determinants change with tumor type, it is imperative to select/test each model for its suitability to mimic best the specific cancer characteristics under investigation. In line with this and to take full advantage of the increased biological relevance of studies performed with 3D microspheroids, distinct projects to generate comparative data of 3D models to support the selection of the appropriate model are needed. For example the IMI-PREDECT consortium (http://www.imi.europa.eu/content/predect) is a project that aims to evaluate advanced in vitro models.

Genetically encoded or chemical sensors allow distinct cell types in multi-cell-type 3D microspheroids to be distinguished and when combined with flow cytometry, separated and molecularly analyzed through the application of genomic, proteomic and metabolic technologies. Fluorescent sensors also permit interrogating activities of specific signal transduction pathways and their transcriptional responses in specific cell types in multi-cell type 3D microspheroids to deduce cancer cell characteristics and mechanisms relevant for tumor growth in vivo. For imaging of 3D microspheres (as compared to 2D monolayer cultures) a high Z-resolution and image depth of microspheres must be reached. Moreover, at single-cell resolution, optical microscopes must be capable of accessing also cells close to the core of the microsphere at high resolution. An image depth of about 1 mm is reachable with current multiphoton microscopy techniques [154]. Image acquisition at high speed as it is required for high-throughput screening remains a formidable task. Irrespective, multi-cell type 3D microspheroids offer unique prospects for novel molecular target and lead compound

identification. In this regard, the development of novel labware for screening of 3D microspheres in standardized micro-plate formats suitable for robotic systems is rapidly moving forward.

A possible limitation of the various existing cancer cell lines and their phenotypical behavior in 3D microspheres might be that the isolation of these cells was originally by growth in 2D monolayer cultures and such a 2D pre-selection might negatively influence the in vivo relevance of such models. Therefore, isolation of primary tumor cells without prior adaption for 2D growth might provide better and more in vivo-like cancer cell lines for studies in 3D microspheres.

Clearly, the field of 3D microsphere technology development and application is rapidly advancing and is beginning to impact enormously both discovery and translational research efforts as they relate to cancer. Moreover, this field holds the promise as a superior system for molecular drug target identification and validation thus facilitating the process of drug development and helping to realize the promise of advancing precision cancer medicines.

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