REVIEW ARTICLE

Three-dimensional cell culture models for anticancer drug screening: worth the effort? †

Eddy-Tim Verjans[1#], Jordi Doijen[1#], Walter Luyten[1], Bart Landuyt[1], Liliane Schoofs[1].

[1] KU Leuven, Department of Biology, Division of Neurobiology and Animal Physiology, Naamsestraat 59, 3000 Leuven, Belgium.

Corresponding author:
E-Mail: EddyTim.Verjans@kuleuven.be
Tel. +32 16 37 37 58,
Fax: +32 16 32 39 02

# E.-T. V. and J.D. contributed equally to this work.

Contract grant sponsor: Innovation by Science and Technology (contract grant number: SBO050164, SBO100042), Research Foundation Flanders (contract grant number: G.0588.11 and GPT-D0605-SB/151105).

Received 12 June 2017; Accepted 13 June 2017
Journal of Cellular Physiology

DOI 10.1002/jcp.26052

Abstract

High attrition of new oncology drug candidates in clinical trials is partially caused by the poor predictive capacity of artificial monolayer cell culture assays early in drug discovery. Monolayer assays do not take the natural three-dimensional (3D) microenvironment of cells into account. As a result, false positive compounds often enter clinical trials, leading to high dropout rates and a waste of time and money. Over the past two decades, tissue engineers and cell biologists have developed a broad range of 3D *in vitro* culturing tools that better represent *in vivo* cell biology. These tools preserve the 3D architecture of cells and can be used to predict toxicity of and resistance against antitumor agents. Recent progress in tissue engineering further improves 3D models by taking into account the tumor microenvironment, which is important for metastatic progression and vascularization. However, the widespread implementation of 3D cell cultures into cell-based research programs has been limited by various factors, including their cost and reproducibility. In addition, different 3D cell culture techniques often produce spheroids of different size and shape, which can strongly influence drug efficacy and toxicity. Hence, it is imperative to morphometrically characterize multicellular spheroids to avoid generalizations among different spheroid types. Standardized 3D culturing procedures could further reduce data variability and enhance biological relevance. Here, we critically evaluate the benefits and challenges inherent to growing cells in 3D, along with an overview of the techniques used to form spheroids. This is done with a specific focus on antitumor drug screening.

Keywords: Three-dimensional cell cultures; monolayer cultures; tumor spheroids; scaffold-based techniques; scaffold-free techniques

Abbreviations

C. elegans, *Caenorhabditis elegans*; CAF, Cancer-associated fibroblasts; CRC, colorectal cancer; DILI, Drug-induced liver injury; DME, Drug-metabolizing enzyme; EC, Endothelial cell; ECM, extracellular matrix; EGFR, Epidermal growth factor receptor; EMT, Epithelial mesenchymal transition; HSG, Human submandibular salivary gland; MCTS, Multicellular tumor spheroid; MLM, Magnetic levitation method; NCP, Nanoculture plate; NSCLC, non-small-cell lung carcinoma; OMCS, Organotypic multicellular spheroids, PEG, Polyethylene glycol; poly-Hema, poly(2-hydroxyethyl methacrylate); R&D, Research and development; RWV, Rotating wall vessel; TDTS, Tissue-derived tumor spheres ;ULA, Ultra-low attachment.

1. Introduction

Pharmaceutical research and development (R&D) has increased significantly over the last decade (Giaccotto *et al*. 2005). It is estimated that it may cost up to 2.6 billion dollar to get a new drug to the market (Avorn 2015). Factors that boosted R&D expenditures are larger clinical trial sizes, enhanced clinical trial complexity and increasingly stringent regulatory landscapes. However, a significant part of expenditure in drug discovery is attributed to high attrition rates of drug candidates in clinical trials (Waring *et al*. 2015). Even though it is possible to avoid false discoveries by adjusting and improving experimental design and practices, high failure rates appear to have a deeper underlying cause. This cause is represented by a large gap between *in vitro* hypotheses and *in vivo* 'veritas'.

In essence, only a small fraction of the incredibly complex nature of living organisms is currently understood by present-day science. To unpick this complexity, researchers rely on a broad range of techniques and model systems that mimic important features of human physiology. Each of these approaches strike a delicate balance between usability, cost, and resemblance to *in vivo* circumstances. In addition, there is a huge demand for faster drug development due to urgent global health changes such as emerging pandemic viruses and the increase in antibiotic resistance (Waring *et al*. 2015).

Cancer is another major public health issue, particularly in the United States and other Western countries (Cunningham and You 2015). As cancer becomes more common, the demand for new anticancer drugs increases. Over the past two decades, large investments in oncology drug development have been made. In comparison with other drugs, more oncology drug failures seem to reach late-stage clinical trials were the expenses are huge (DiMasi and Grabowski 2007). This is partially due to the fact that preclinical studies lack efficiency when it comes to identifying molecules that can alter the outcome of cancer development and progression (Ocana *et al*. 2010).

Generally, standard preclinical screening procedures for anticancer agents involve target identification of a compound on immortalized cell lines cultured in 2D. Once a target has been identified, experiments of different complexity are performed using computational, *in vitro*, and *in vivo* models (Hughes *et al*. 2011). During this process, each technique or model system suffers from inherent limitations by which promising candidate drugs can be missed or by which adverse drug side effects can be overlooked.

In vivo model systems such as *Caenorhabditis elegans* (*C. elegans*), *Drosophila melanogaster*, and *Rattus norvegicus* are commonly used to expand our understanding of similar physiological processes in humans. For example, Beets *et al*. used *C. elegans* as a neurobiological model to study neuropeptidergic signaling and discovered that vasopressin/oxytocin-related peptides play a key role in the associative learning behavior of the roundworm. Interestingly, vasopressin and oxytocin also regulate cognitive processes in mammals, confirming the presumption that biochemical processes in the nervous system of *C. elegans* are similar to organisms of higher complexity (Beets *et al*. 2012). Kobet *et al*. report that several oncogenic signaling pathways (Wnt, Notch, and Ras) in *C. elegans* are highly conserved in vertebrates, including humans (Kobet *et al*. 2014). In addition, many genes known to be involved in human cancer have functional counterparts in well-characterized model organisms (Kobet *et al*. 2014; Cunningham & You 2015; Walrath *et al*. 2010). Studying these genes can provide insight into the molecular mechanisms underlying the formation of cancer.

However, the inadequacy of most *in vivo* models for predicting the clinical outcome of a drug candidate in humans is a recurrent issue. Experimental data from animal tests can be limited by interspecies differences which are likely to be theoretically and technically difficult to overcome. Knight elaborated on this issue and proposes a ban on using animal models that lack scientific evidence establishing human predictivity or utility. This fact, in conjunction with strong ethical concerns, prompted research institutions to develop and implement alternative models to diminish animal testing (Knight 2008).

Numerous *in vitro* test methods have been developed based on human cell and tissue cultures. The majority of cell-based assays rely on the growth of cells as immortalized 2D monolayers, which are easy to work with and allow for simple and efficient culturing workflows. 2D cell cultures serve as the main workhorse for basic cellular research and are being used for predictions of drug activity, metabolism and toxicity *in vivo* (Antoni *et al*. 2015). For oncology drug screening, cytotoxicity assays are typically performed on established tumor cell lines grown in monolayer cultures since they grow uncontrolled and rapidly. In addition, transformed cell lines are used. These cell types often do not resemble the native cellular function found in normal primary cells, but instead are selected for a

particular research purpose such as increased proliferative capacity or higher plating efficiency. Transformed cells have acquired a transient or permanent change in their phenotype by genetic changes, which may diminish their capacity as a model to predict the *in vivo* performance of a drug candidate. These type of cultures undergo genetic drift and therefore undergo unwanted genetic changes overtime (Kimlin *et al*. 2013).

To better understand native cellular function, primary cells are often used in cell-based research programs. Primary cultures are directly derived from living tissue and are therefore more likely to reflect the properties of native cells *in vivo*. However, primary cells have a limited lifespan, can quickly dedifferentiate, and the preparation and culture workflows are much more challenging compared to permanent cell lines (Gordon *et al*. 2013). Moreover, primary cells often have to be isolated from a heterogeneous cell population, in order to study the cell type of interest (Gordon *et al*. 2013).

The introduction of co-cultures, where multiple cell types are grown together in the same culture dish, represent a higher degree of *in vivo* resemblance compared to monocultures. Both immortalized cell lines and primary cells can be cultured in co-culture systems. These mixed populations can be used to study cell-cell interactions *ex vivo* (Miki *et al*. 2012). Nevertheless, analyzing cellular interactions remains a major challenge, especially since these interactions tend to change in different environments and some interactions only occur within defined pH ranges (Goers *et al*. 2014).

All the aforementioned model systems, ranging from permanent 2D monocultures or primary cultures to co-culture approaches have one common drawback: cells are typically cultivated on plastic dishes or tissue culture flasks. Culturing cells on a polystyrene surface has serious limitations, as the cells adhere to an unnatural plastic substrate. The polystyrene surface is often pre-coated in order to help the cells attach to the substrate. However, this configuration inadequately represents the complex physiological 3D environment where cells interact with each other and with the extra cellular matrix (ECM) and have a specific cellular organisation. Therefore, these systems fail to resemble the complex nature and heterogeneity of clinical tumors. As a consequence, monolayer cultures can lose the capacity to respond to oncology drugs in a relevant manner (Lee *et al*. 2007).

The poor resemblance of *in vivo* architecture could lead to dubious conclusions and can endanger subsequent R&D efforts. For example, Gomez-Roman *et al*. evaluated whether glioblastoma-derived cells responded to radiotherapy more strongly when it was given in combination with molecular targeted agents for which clinical data was available. While the Epidermal Growth Factor Receptor

(EGFR) antagonist Erlotinib enhanced the radiosensitivity of cells cultured in 2D, it had no efficacy in phase II clinical trials (Gomez-Roman *et al*. 2017). This does not necessarily mean that 2D models are wrong *per se*, as clinical failure could also be due to pharmacokinetic variability. However, this study underlines the need for better predictive *in vitro* models. Multiple studies suggest that drug responses of 2D cell cultures poorly predict the outcome of clinical studies (Gomez-Roman *et al*. 2017; Aljitawi *et al*. 2014; Edmondson *et al*. 2014; Ravi *et al*. 2015; Hingorani *et al*. 2009; Weaver *et al*. 1997; Bhadriraju and Chen 2002; Guengerich 2007; Ramaiahgari *et al*. 2014).

A promising approach to deal with high failure rates in clinical trials is the development of 3D cell cultures. 3D models often closely reflect cell behavior in living tissues and tumors (Antoni *et al*. 2015; Yamada and Cukierman 2007). They possess many features by which they resemble *in vivo* tumors including physiologically relevant cell-cell and cell-ECM interactions, hypoxia and central necrosis, and drug resistance (Antoni *et al*. 2015; Fayad *et al*. 2009; Baker and Chen 2012; Wartenberg *et al*. 2003). In addition to molecular drug resistance, spheroid cultures can help predict drug penetration, an important cause of resistance in tumors that is often overlooked (Minchinton and Tannock 2006). Due to these features, the spheroid model is often seen as a more stringent and representative platform for *in vitro* drug screening (Thoma *et al*. 2014). Therefore, it might be useful to implement 3D studies in drug screening programs to support monolayer findings before advancing to animal testing. Such an approach might ensure more efficient animal testing and may reduce the number of animals used. However, 3D models cannot completely replace animal research as they still underappreciate the complex nature of living organisms.

As powerful as they are, some clear limitations prevent the integration of 3D cell culture models into mainstream drug discovery pipelines. Different 3D culturing techniques can be used to form spheroids, which have an impact on various spheroid parameters including size, shape, density, surface features, and internal textures (Härmä *et al*. 2014). Differences in spheroid configuration can in turn affect the outcome of drug delivery and efficacy studies. Consequently, there is a critical demand for an improved morphometric characterization of multicellular spheroids, which can reduce the variability between different experimental setups and increase the comparability of results.

In this review, the advantages and limitations inherent to 3D cell culture models are discussed in more detail. Additionally, commonly used techniques to maintain the 3D structure of a cell are described.

2. Merits and demerits of 3D cell culture

3D cell culture models can be created by growing cells into larger 3D cell aggregates (spheroids) *via* a wide variety of scaffold and scaffold-free techniques. One of the main objectives of culturing 3D spheroids *in vitro* is to examine the pharmacodynamics of drug candidates before carrying out clinical trials (Griffith and Swartz 2006). Additional applications of 3D cell cultures include studying cancer, pharmacology, cell differentiation, and tissue engineering (Antoni *et al*. 2015; Edmondson *et al*. 2014; Ravi *et al*. 2015; Bhadriraju and Chen 2002).

A powerful improvement of tumor spheroids over conventional 2D cell cultures is the presence of metabolic and proliferative gradients across their spherical geometry that can influence pharmacological efficacy. Spheroids of permanent cell types that reach sizes of 500 µm or more often undergo central necrosis. This necrosis evolves because nutrient and oxygen supply is limited in the center, the pH is low and there is an accumulation of waste. A viable layer of approximately 200 µm surrounds the outer surface of the necrotic core (Acker *et al*. 1987; Carlsson and Acker 1988). Before undergoing cell death, cells located more in the center adapt their metabolism and become quiescent in order to maintain homeostasis (Walenta *et al*. 1990). This leads to the typical zonation found in spheroids with proliferating cells found on the outside of the spheroid, whereas quiescent and necrotic cells are harbored within the spheroid (figure 1). Similarly, solid tumors often have regions with different proliferation rates and regions with mild to severe oxygen deficiencies, due to the lack of blood supply to growing tumor nodules (Mehta *et al*. 2012; Strese *et al*. 2013; Lin and Chang 2008). Therefore, spheroid models mimic avascular tumors and can be used to predict the efficacy of radiotherapy and chemotherapy and to predict the metastatic potential of certain tumors.

The value of working with 3D tumor models was highlighted by a study of Tung *et al* (Tung *et al*. 2011). Two anticancer drugs, 5-fluorouracil (5-FU) and tirapazamine, with different modes of action produced distinct responses in 3D spheroids compared to conventional 2D cell cultures of human epithelial carcinoma cells. 5-FU is a well-characterized compound that inhibits cell proliferation, whereas tirapazamine is an anticancer drug that functions as a hypoxia-selective cytotoxin (Tung *et al*. 2011; Longley *et al*. 2003). The 3D spheroids remained viable after 5-FU treatment, whereas cells grown as 2D monolayers did not survive the treatment. Because 2D cell cultures initially proliferate at a relatively uniform rate across the plastic substrate, 5-FU is able to exert its DNA-damaging effect. Instead, spheroids have a lower proliferation rate, leading to a reduced sensitivity to 5-FU.

At low oxygen levels, tirapazamine is activated and forms a toxic radical that is exclusively effective on cells cultured in 3D since they exhibit hypoxic regions inside the spheroid that promote radical formation (Denny and Wilson 2000). Hypoxia in the center also stalls proliferation, indirectly making spheroids less susceptible to anti-proliferative drugs such as cisplatin (Mehta *et al*. 2012). Fayad *et al*. demonstrated that colorectal cancer HCT116 cells grown in 3D are indeed less susceptible to cisplatin than their monolayer counterparts (Fayad *et al*. 2009). Testing for synergistic effects of drugs that target hypoxic zones like tirapazamine and conventional anticancer drugs such as cisplatin may provide new strategies to oncology drug development. The former results show that the development of such strategies can potentially be improved using spheroid cultures.

Differential drug responses of cells grown in 3D vs 2D are not solely related to differential zones of proliferation or oxygen availability. Multiple studies report differences in gene and protein expression between 3D spheroids and 2D cultures that change drug efficacy, metabolism, and cell communication (Aljitawi *et al*. 2014; Edmondson *et al*. 2014; Ravi *et al*. 2015; Ramaiahgari *et al*. 2014; Loessner *et al*. 2010; Gaskell *et al*. 2016; Takahashi *et al*. 2015; Sakai *et al*. 2010; Olsavsky *et al*. 2007; Jeon *et al*. 2016; Wiśniewski *et al*. 2016). Aljitawi *et al*. showed that an increase in N-cadherin expression in spheroid co-cultures of leukemic and bone marrow mesenchymal stem cells reduces chemotherapy efficacy when compared to monolayer co-cultures. The precise role of *N*-cadherin in mediating leukemic cell resistance is currently not well understood but may be an interesting target for leukemia therapy (Aljitawi *et al*. 2014).

Monolayer cultures of hepatocytes quickly dedifferentiate and lose many characteristics of *in vivo* liver tissue such as the secretion of albumin (Ramaiahgari *et al*. 2014; Olsavsky *et al*. 2007; Jeon *et al*. 2016; Wiśniewski *et al*. 2016). This limits their value in predicting drug-induced liver injury (DILI). Different groups have shown, using both protein and RNA expression studies, that various liver-specific functions are re-acquired when growing cells in spheroid cultures (Ramaiahgari *et al*. 2014; Gaskell *et al*. 2016; Takahashi *et al*. 2015; Jeon *et al*. 2016). Of particular interest is the upregulation of metabolic genes in spheroid cultures as compared to hepatic cells cultured in 2D. The expression level of drug-metabolizing enzymes (DMEs) in spheroids more closely resembles the expression level of DMEs in native liver tissue, suggesting that spheroids are more adequate *in vitro* models than monolayer cultures to study the metabolic breakdown of drugs (Ramaiahgari *et al*. 2014; Olsavsky *et al*. 2007; Wiśniewski *et al*. 2016). DMEs are important for solubilizing drugs by means of hydroxylation and conjugation, which facilitates their transport and clearance. The biotransformation of drugs by the cytochrome P450 system often results in the production of reactive intermediates (Lakehal *et al*. 1999). It is known that many drugs are initially inert but exert toxic side effects when

they are converted to their metabolites by DMEs. Many drugs withdrawn from the market produce these types of reactive intermediates (*e.g*. benoxaprofen) emphasizing the necessity to find new, convenient and more accurate systems for hepatotoxicity testing (Guengerich 2007).

Loessner *et al.* measured mRNA levels in ovarian cancer cells in 2D and 3D cultures and reported increased expression of receptors for $\alpha_3/\alpha_5/\beta_1$ integrins and matrix metalloprotease 9 (*MMP9*) in 3D cultures compared to cells grown in 2D monolayers (Loessner *et al*. 2010). Colorectal cancer (CRC) 3D spheroids showed altered EGFR expression as compared to monolayer cultures which impaired the efficacy of pharmacological EGFR inhibition (Luca *et al*. 2013). In melanoma cells, genes encoding laminin, hyaluronic acid, CXCL1, IL-8, as well as pro-angiogenic genes were upregulated in 3D spheroids compared to conventional 2D monolayer cultures. These genes are associated with melanoma progression *in vivo* (Ghosh *et al*. 2005). Maria *et al*. discovered that spheroid cultures of human submandibular salivary gland (HSG) cells increased the secretion of acinar proteins. This increase was not associated with enhanced transcription of the according genes, but rather a consequence of translational regulation. In this case, cells cultured in 2D experienced a protein translation defect, leading to a lower acinar protein production (Maria *et al*. 2011). Stimulation of Fas/CD95 which is highly toxic *in vivo,* induces apoptosis in the hepatocyte cell line MhAT3F 3D spheroid model but is not effective in monolayer cultures due to increased NF-κB signaling downstream of the engaged death receptor (Haouzi 2005). These and many other reports indicate that changes in gene expression and proliferation can drastically alter the experimental outcome and thus determine whether a pharmaceutical compound is seen as effective or not.

Spheroid morphology and organization also affect cellular processes such as apoptosis and histone acetylation (Tibbitt and Anseth 2009). Morphology changes allow spheroid cultures to better represent the *in vivo* situation than 2D cultures in which cells flatten and proper cell-to-cell interactions are virtually eliminated (Zhang *et al*. 2005; Griffith and Swartz 2006). For instance, cell polarization in spheroids is suggested to be more accurate than in monolayer cultures (Antoni *et al*. 2015). This could be of particular interest to study the behavior of polarized epithelial cell cultures (MacNeil 2007).

Taken together, growing cells in a 3D environment changes cell contacts and nutrient availability which in turn alters the expression of genes. Differences in gene expression affect different physical and physiological properties including metabolism and proliferation. It also influences different types of cell behavior such as cell migration, differentiation, and communication (Antoni *et al*. 2015; Edmondson *et al*. 2014; Ravi *et al*. 2015). The gene expression profiles of cells grown in 3D are often more similar to expression patterns found in native tissues or primary tumor samples (Antoni *et al*.

2015; Edmondson *et al*. 2014; Ravi *et al*. 2015; Yoshii *et al*. 2011; Magdeldin *et al*. 2014). Multiple studies compared protein or gene expression levels of liver monolayer cultures with liver tissues or spheroid cultures and suggest that expression patterns of spheroids more closely resemble those of native liver tissue (Ramaiahgari *et al*. 2014; Takahashi *et al*. 2015; Olsavsky *et al*. 2007; Wiśniewski *et al*. 2016). Cell-cell interactions that take place in tumor tissue and hypoxia play an important role in radioresistance and drug resistance (Santini and Rainaldi 1999; Mueller-Klieser 1997; Olive and Durand 1994; Durand and Olive 1992). These tumor characteristics are also found in spheroid cultures which make them powerful tools for improved drug discovery. For instance, they can be used to eliminate potential drug candidates that are amenable to hypoxia-induced drug resistance. In addition, they can help solve therapeutic problems related to metabolic and proliferative gradients.

Despite these promising findings, none of the 3D methods have been implemented in cell-based research programs on a large scale. This is due to their high costs, along with difficulties to create uniformly sized spheroids and develop spheroid co-cultures (Edmondson *et al*. 2014; Mehta *et al*. 2012). Commercially available assay formats are not always optimized for 3D cell culturing and data interpretation can be challenging due to the lack of standardized protocols (Antoni *et al*. 2015). In contrast to 2D cell cultures, researchers can also not rely on a rich scientific literature addressing mechanisms of drug interactions, cell differentiation, and cell signaling in a 3D environment.

Several studies suggest that a thorough characterization of the spheroid system is required to avoid generalizations among different types of spheroids (Katt *et al*. 2016). For example, Mellor *et al*. examined the effect of different antitumor drugs including cisplatin, vinblastin and doxorubicin on non-small-cell lung carcinoma (NSCLC)-derived spheroids and discovered that there were big differences in drug efficacy of the different drugs. More striking, they found that the same drugs had different efficacies depending on the proliferative status of the spheroids (Mellor *et al*. 2005). This variability makes it difficult to compare independent drug exposure studies on spheroids. A similar observation was made by Zanoni *et al*., who showed that 3D cell cultures heterogeneous in shape and volume may respond differently to chemical or physical treatments. They developed open-source software capable of automatically analyzing several morphological parameters of spheroids, thereby reducing data variability and enhancing the biological relevance (Zanoni *et al*. 2015). Mathematical models can also help simulating dynamics of drug distribution within spheroids, as exemplified by Mehta *et al* (Mehta et al. 2012). These models predict gradients of oxygen, nutrients, lactate and glucose, as well as the extent and location of quiescent cells in spheroids. This enables researchers to predict the effectiveness of drug treatments.

In addition, 3D cell culture models are not being used as a routine tool in preclinical cancer research since the *in vivo* multicellular complexity and microenvironment of tumors is not fully recapitulated using these models. Tumors do not function as solitary units. Their microenvironment often harbors different types of cells that contribute to tumorigenesis in a specific way. For instance, endothelial cells (ECs) can vascularize tumors after undergoing an angiogenic switch (Verbridge *et al*. 2013). Vasculature enhances the tumor's nutrient and oxygen availability and thus influences tumor growth, survival and metastasis *in vivo* (van Duinen *et al*. 2015).

In an effort to better recapitulate the *in vivo* situation, 3D co-cultures have been developed. Co-cultures usually combine tumor cells with stromal cell types such as ECs or cancer-associated fibroblasts (CAFs) (Friedrich *et al*. 2009). For instance, a monolayer of ECs can be used as a surface to seed 3D spheroids onto. In this manner, a simple vasculature-containing tumor model is achieved. Implementing a vascular component in 3D cell culture models is important since blood vessels can create oxygen gradients that in turn can lead to endothelial sprouting and promote cell migration via chemotaxis (Verbridge *et al*. 2013; Mosadegh *et al*. 2015). In addition, implementing vasculature is important for evaluating anti-angiogenic compounds since they can elicit tumor adaptation and increase the invasiveness of the tumor (Pàez-Ribes *et al*. 2009).

Another typical feature of many tumors is their ability to undergo epithelial mesenchymal transition (EMT). This mechanism induces metastatic dissemination of tumor cells which can create variability in the tumor population. This in turn can lead to a decrease in drug efficacy, as demonstrated by Mani *et al* (Mani *et al*. 2008). In 2012, 8 million people died of cancer and metastasis appeared to be the major cause of death (Torre *et al*. 2015). Even though metastatic progression has an enormous impact on disease outcome, the process is still not fully understood. Therefore, it is crucial to develop improved models that better reflect metastatic features.

Kalluri *et al*. report that CAFs are involved in all stages of cancer progression, including metastasis (Kalluri 2016). CAFs are a major component of cancer stroma and contribute to disease progression by secreting growth factors, cytokines and chemokines, as well as modulating angiogenesis. Various research groups use co-cultures of tumor spheroids with CAFs to examine the effect of CAFs on breast and colorectal cancer progression (Li and Lu 2011; Jaganathan *et al*. 2014; Kim *et al*. 2015; Jeong *et al*. 2016). Li and Lu demonstrated that CAFs strongly influence spheroid morphology in breast cancer cells (Li and Lu 2011). Kim *et al*. showed that colorectal spheroids co-cultured with CAFs mimic the EMT-state of the invasive margin of early metastatic native tumors (Kim *et al*. 2015). This further underscores the importance of fibroblasts in EMT and thus in tumorigenesis in general. Jeong *et al.* described the use of a collagen-matrix chip-based system with imbedded microfluidics to

investigate colorectal cancer spheroids progression in the presence of CAFs. They showed that CAFs enhance HT-29 spheroid growth and contribute to spheroid drug resistance to paclitaxel (Jeong *et al*. 2016). Jaganathan *et al*. used the magnetic levitation technique to set up co-cultures of breast cancer spheroids with CAFs. They report that this spheroid model closely mimics the native tumor environment. In addition, large-sized spheroids are obtained within 24 hours which can be used for drug efficacy tests (Jaganathan *et al*. 2014).

Recently, an heterogeneous scaffold-based 3D cell culture model was developed to study tongue tumorigenesis. In this system, normal, dysplastic as well as malignant tissues are included. Sawant *et al*. showed that stromal fibroblasts were required to accurately mimic cell proliferation and differentiation of native tumor tissue and that these fibroblasts play an important role in maintaining cell-cell adhesion (Sawant *et al*. 2016).

To conclude, 3D models have proven to be useful for evaluating adverse side effects and efficacy of antitumor drugs at an early stage in the drug discovery pipeline and can contribute to present-day methodologies used in preclinical studies. Improvements in 3D cell culture models overcome barriers related to reproducibility, costs, and cumbersome procedures. However, the lack of standardization limits their implementation in cell-based drug screening programs. In addition, the majority of the developed 3D models do not include a vascular component. Therefore, these models cannot be used to study tumor vascularity and instead can solely be implemented in avascular tumor research.

3. Scaffold and non-scaffold techniques

Tissue engineers and cell biologists have been trying to mimic the highly complex 3D arrangement of cells for many years. An ideal system should be both easy and fast when it comes to culturing and analysis (Friedrich *et al*. 2009). Weiswald *et al*. suggest that there are four spherical tumor models in which cancer can be studied, *i.e.* tumorospheres, tissue-derived tumor spheres (TDTS), organotypic multicellular spheroids (OMCS), and multicellular tumor spheroids (MCTS). This classification is based on differences in sphere biology and culturing techniques for obtaining multicellular spheres. Tumorospheres are generally used as a model of cancer stem cell expansion and established in serum-free culture medium supplemented with growth factors in low-adherent conditions, whereas TDTS can be obtained by cancer cells after partial dissociation of tumor tissues and subsequent remodeling and compaction. OMCS can be achieved by by culturing a part of tumor tissue in a dish. The cultured tissue rounds up and forms an organotypic multicellular spheroid. Spheroids can also be formed by aggregation of suspended cells followed by compaction in low-binding conditions. In this manner, MCTS are obtained (Weiswald *et al*. 2015). We focus on MCTS since they are balanced

between complexity and reproducibility and can potentially be useful in preclinical procedures for anticancer agents.

In general, there are two methods that can be used for MCTS formation: scaffold techniques and scaffold-free techniques. A schematic overview of the most common 3D culturing techniques applied to grow spheroids is provided in figure 2. 3D scaffolds either rely on biological or synthetic polymers. This microarchitecture reflects the native ECM composition and can provide a biologically active microenvironment for the cells to interact with each other, to proliferate, and to migrate (Griffith and Swartz 2006; Mehta *et al*. 2012). Biological scaffolds are created from a range of biological components such as agarose, laminin, collagen, vitronectin, fibronectin, and gelatin. Cells can be dispensed in a microwell previously coated with a particular scaffold, or hydrogel-based matrices can be used.

Hydrogels are natural or synthetic networks of crosslinked polymer chains that possess elevated water content, which facilitate nutrient, oxygen, and waste transport. Hydrogels derived from a natural origin are highly bioactive due to the presence of a wide variety of endogenous molecules, which can be advantageous for many cellular processes including proliferation or differentiation. One of the most commonly used naturally-derived hydrogels is the so-called Matrigel, which is composed of a mixture of ECM proteins secreted by Engelbreth-Holm-Swarm sarcoma cells (Bachmann *et al*. 2015). However, such scaffolds are often not well characterized making it challenging to identify the endogenous factors that promote a certain cell behavior. Biological hydrogels may also contain unknown or unwanted components which limits their use for clinical work. On the other hand, hydrogels composed of synthetic polymers such as polyethylene glycol (PEG) are relatively well characterized, highly reproducible, and easily manufactured, but lack the bioactive endogenous molecules that can be beneficial for cell function (Tibbitt and Anseth 2009).

Due to recent progress in micro-fabrication, micropatterned surface microplates were developed. These plates can be optimized for spheroid formation and cell networking events by imbedding different possible micro-configurations in the bottom. One of the latest micropatterned 3D cell culture products was engineered by Organogenix, Inc. (Woburn, MA, USA). Seeded cells attach to the nano-scale structure plastic film on the well-bottom surface of the so-called NanoCulture Plate (NCP). On the bottom, a structure is present that mimics the normal ECM (Yoshii *et al*. 2011; Aritomi *et al*. 2014). Because cells do not adhere strongly to the microstructure on the culture surface, they start migrating and reorganize into 3D structures.

Even though NCPs do not overcome all of the above mentioned disadvantages (*e.g.* lack of vasculature, expensive) inherent to working with 3D cell cultures, they overcome flaws of other scaffold-type 3D culture systems such as lot-to-lot variation, difficulty of imaging, and cumbersome culturing workflows (Yoshii *et al*. 2011). Growing spheroids using NCPs is also suitable for high-throughput screening and offers great potential for future integration into contemporary drug screening procedures (Horman *et al*. 2015).

Scaffold-free methods have also been devised to generate 3D cell cultures. The best known example hereof is the so-called hanging drop method, in which single cells are placed in a hanging drop culture and incubated under physiologically relevant conditions until they form 3D spheroids (Foty 2011; Kelm *et al*. 2003). This technology is based on the fact that cells self-assemble into 3D structures in the absence of a surface to which they can adhere. This is technically achieved by creating plates with a small opening at the bottom of the well, thereby enabling droplet formation of the culture medium. This droplet enables the cells to form multicellular spheroids. Surface tension prevents the droplets from being displaced during experimental manipulation. Using this method, Messner *et al.* successfully obtained spheroids of hepatocytes that exerted hepatic functions such as glycogen storage and bile canaliculi formation (Messner *et al*. 2013). The hanging drop technique allows easy control of spheroid size, but is relatively low throughput (Katt *et al*. 2016).

Liquid overlay spheroid cultures are scaffold-free cultures that use microplates with agar/agarose or poly(2-hydroxyethyl methacrylate coatings to prevent cell attachment to the surface. This forces cells into a suspended state, enabling the formation of 3D multicellular spheroids (Rotem *et al*. 2015). Ivanov *et al*. used these ultra-low attachment (ULA) spheroid microplates to monitor growth kinetics and drug toxicity of neuronal stem cells (Ivanov *et al*. 2014). Vinci *et al.* demonstrated that the ULA-approach compares closely with conventionally generated 3D spheroids, but with the additional advantage that automated analysis is easier (Vinci *et al*. 2012). However, according to Katt *et al.*, experiments that require long-term cultivation of the 3D spheroids can be difficult using this approach (Katt *et al*. 2016).

In the scaffold-free Magnetic Levitation Method (MLM) cells are bound by a nanoparticle overnight in order to make them magnetic. The cells are then resuspended in medium and a magnetic field is applied in such a way that the cells concentrate at the air-liquid interface and form aggregates. 3D spheroids can be obtained in less than 16 hours, which is faster compared to other scaffold-based cultures. In addition, there is no need for specialized medium or an artificial substrate (Haisler *et al*. 2013). Hau *et al*., used this approach to form spheroids of the colon cancer LoVo cells and showed

that these 3D cultures were more resilient to anticancer therapy than corresponding monolayer cultures (Hau *et al*. 2016).

All scaffold-based culturing systems, as well as the hanging-drop and the liquid overlay system, have one common drawback: the medium is stagnant which can cause problems related to nutrient supply and waste disposal. This is especially disadvantageous when growing spheroids over longer periods of time. For the MLM, some magnet based techniques can be used to facilitate medium exchange or spheroid handling e.g. MagPen™ from n3D Biosciences.

For long-term cultivation, non-stagnant culture systems such as the spinner flask are often more suitable. Within the spinner flask, cells lack a substrate and as a result they interact with each other and form aggregates. Spheroids can also be pre-formed using a non-adherent initiation dish, a so called mother dish, which is meant for short term cultivation. After initiation, the spheroids are transferred into the spinner flask. The spinner flask already exists for over 30 years but remains one of the most efficient scaffold-free systems for obtaining large amounts of spheroids within a particular size range under well controlled nutrient supply. Other commonly used non-stagnant systems are roller tubes and the gyratory shaker (Friedrich *et al*. 2009).

More recently, bioreactor systems were developed that provide efficient mass transfer and automated control of temperature, pH, and other environmental factors (Hickman *et al*. 2014). An example is the rotating wall vessel (RWV) or NASA bioreactor that allows for continuous medium perfusion and a well-controlled nutrient flow rate. In this rotary system, cells are kept in suspension under very low sheer stress by microgravity simulation (Ingram *et al*. 1997). Since the RWV is more expensive than the spinner flask and since it has a less straightforward setup, the advantages are limited.

The demand for systems that adequately mimic the *in vivo* environment of cells led to the development of 'body-on-a-chip' systems. Their resemblance with functional tissue is achieved by imbedding the latest generation microfluidics in order to enhance perfusion of the microenvironment. These systems are further improved by exposing the cells to matrix proteins such as collagen or Matrigel so that the system meets the spheroid's need for nutrients, oxygen and waste removal (Hickman *et al*. 2014; Jang *et al*. 2015). These systems hold great promise for long-term cultivation and repeated *in vitro* drug exposure studies.

A serious drawback of all rotating systems and systems with perfusion is that they require high quantities of medium while in drug testing it is often preferred to use low amounts of candidate drugs (Friedrich *et al*. 2009). Therefore, drug exposure tests might be better of using stationary

systems such as the ones described previously. Perfusion-based systems are rather positioned in between high-throughput *in vitro* screening and *in vivo* preclinical models.

Albittron and Miller provide a good technology overview of different bio-printing methods that can be used to fabricate 3D scaffolds that better reflect the tumor micro-environment (Albritton and Miller 2017). During 3D bio-printing, bio-material consistent of cells and other bioactive compounds is distributed in 2D patterns and stacked together to form complex 3D structures that can comprise different cell types. In this manner, a more accurate representation of the *in vivo* situation is achieved compared to conventional 2D and 3D cell cultures.

In conclusion, many different approaches exist that allow for spheroid formation. Some systems are better for obtaining large amounts of spheroids or large-sized spheroids, others are better in maintaining nutrient supply and waste disposal and yet others are better for culturing spheroids over longer periods of time.

Since the spheroid's size, shape and environment strongly depend on the culturing system, it is pivotal to develop standardized protocols before spheroid systems can be implemented in the drug discovery pipeline. These protocols will limit the variability between different experimental setups and thus will increase the comparability of the results. In-depth studies must be performed to determine which culturing system is best suited for a particular application. To determine which system is best for evaluating antitumor therapeutic efficacy, different factors have to be considered including throughput, cost, spheroid size, size distribution, and shape. When independent studies employ the same experimental setup, it becomes more meaningful to compare spheroid parameters such as volume growth, viability, survival, migration, and pathophysiological status. Once a standardized 3D system gets widely accepted for therapeutic efficacy determination, it can add significant value to the drug discovery chain as it can give determinative information that is often missed by conventional *in vitro* and *in vivo* drug screening models.

4. Conclusions

Scientists have been looking for models to bridge the gap between *in vitro* findings and *in vivo* relevance for a long time. 3D models offer great promise as intermediate models between conventional 2D cultures and *in vivo* animal experimental models. In 3D cell cultures, cells form multicellular spheroids that re-acquire many features of native tissues or tumors. Their organization limits unnatural cell-synthetic material contacts. 3D spheroids have proven to be useful in many areas of biology, including studies of drug discovery, differentiation, cell proliferation, gene and protein expression, apoptosis, and pharmacology. Currently, one can choose among a variety of 3D

culture methods, each characterized by its advantages, but also by a series of limitations. The current culturing systems are improving and will continue to improve due to technological innovations of media supplements, ECM-like materials, microfluidic devices, and nanoscale coatings. However, in order for these models to translate in more effective drug discovery programs, standardized protocols must be developed employing one suitable culturing system. This will improve result comparability, will lower the complexity of working with 3D systems, and will help prevent drawing false conclusions from independent studies.

For antitumor drug screening, synthetic 3D stationary systems hold great promise since they 1) correlate well to the *in vivo* situation, 2) they are amenable for automated analysis, 3) they have low lot-to-lot variation, 4) they do not require a large amount of material, 5) they can be performed in high-throughput, and 6) they are often compatible with imaging techniques. However, one must also be aware of the limitations of stationary systems. For example, they often lack challenging-to-model features such as a vascular component. Therefore, these 3D cell culture models only mimic avascular tumors and cannot be used to study the role of angiogenesis in tumor growth.

The discovery that the tumor microenvironment plays an important role in differentiation, metastasis and tumor growth has led to tremendous efforts in the field of in tissue engineering. Many heterogeneous models are being investigated and seem to improve *in vivo* alike tumor characteristics. Progress in the field of vasculature and fibroblast implementation in combination with the development of improved microfluidic tools can help improve contemporary 3D cell culture models and make them increasingly interesting for preclinical cancer research. However, a trade-off exists between *in vivo* resemblance and reproducibility as the enhanced complexity of implementing these features further decreases the reproducibility and makes result comparison increasingly challenging.

Conflict of interest statement

None of the authors of this paper have a conflict of interest to declare.

Acknowledgments

This work was supported by the Research Foundation Flanders (FWO; project G.0588.11 and GPT-D0605-SB/151105), and the Agency for Innovation by Science and Technology (IWT; projects SBO050164 and SBO100042). Liliane Schoofs and Walter Luyten are partners in the 'Ageing with elegans' Horizon 2020 project (Grant agreement No 633589).

References

Acker, H., Carlsson, J., Mueller-Klieser, W. & Sutherland, R.M. 1987. Comparative pO2 measurements in cell spheroids cultured with different techniques. *Br J Canc*, 56(3): 325–327.

Albritton, J.L. & Miller, J.S. 2017. 3D bioprinting: improving in vitro models of metastasis with heterogeneous tumor microenvironments. *Dis Model Mech*, 10(1): 3–14.

Aljitawi, O.S., Li, D., Xiao, Y., Zhang, D., Ramachandran, K., Stehno-Bittel, L., Van Veldhuizen, P., Lin, T.L., Kambhampati, S. & Garimella, R. 2014. A novel three-dimensional stromal-based model for in vitro chemotherapy sensitivity testing of leukemia cells. *Leuk Lymphoma*, 55(2): 378–391.

Antoni, D., Burckel, H., Josset, E. & Noel, G. 2015. Three-dimensional cell culture: a breakthrough in vivo. *Int J Mol Sci*, 16(3): 5517–5527.

Aritomi, K., Ishitsuka, Y., Tomishima, Y., Shimizu, D., Abe, N., Shuto, T., Irikura, M., Kai, H. & Irie, T. 2014. Evaluation of three-dimensional cultured HepG2 cells in a nano culture plate system: an in vitro human model of acetaminophen hepatotoxicity. *J Pharmacol Sci*, 124(2): 218–229.

Avorn, J. 2015. The $2.6 billion pill--methodologic and policy considerations. *N Engl J Med*, 372(20): 1877–1879.

Bachmann, A., Moll, M., Gottwald, E., Nies, C., Zantl, R., Wagner, H., Burkhardt, B., Sánchez, J.J.M., Ladurner, R., Thasler, W., Damm, G. & Nussler, A.K. 2015. 3D Cultivation Techniques for Primary Human Hepatocytes. *Microarrays (Basel, Switzerland)*, 4(1): 64–83.

Baker, B.M. & Chen, C.S. 2012. Deconstructing the third dimension - how 3D culture microenvironments alter cellular cues. *J Cell Sci*, 125(13): 3015–3024.

Beets, I., Janssen, T., Meelkop, E., Temmerman, L., Suetens, N., Rademakers, S., Jansen, G. & Schoofs, L. 2012. Vasopressin/oxytocin-related signaling regulates gustatory associative learning in C. elegans. *Science (New York, N.Y.)*, 338(6106): 543–545.

Bhadriraju, K. & Chen, C.S. 2002. Engineering cellular microenvironments to improve cell-based drug testing. *Drug Discov Today*, 7(11): 612–620.

Carlsson, J. & Acker, H. 1988. Relations between ph, oxygen partial pressure and growth in cultured cell spheroids. *Int J Cancer*, 42(5): 715–720.

Cunningham, D. & You, Z. 2015. In vitro and in vivo model systems used in prostate cancer research. *J Biol Methods*, 2(1): 17–28.

Denny, W.A. & Wilson, W.R. 2000. Tirapazamine: a bioreductive anticancer drug that exploits tumour hypoxia. *Expert Opin Investig Drugs*, 9(12): 2889–2901.

DiMasi, J.A. & Grabowski, H.G. 2007. Economics of new oncology drug development. *J Clin Oncol*, 25(2): 209–216.

Durand, R.E. & Olive, P.L. 1992. Tumour cell kinetics and heterogeneity: insights from multicell spheroids. *BJR supplement*, 24: 79–83.

Edmondson, R., Broglie, J.J., Adcock, A.F. & Yang, L. 2014. Three-Dimensional Cell Culture Systems and Their Applications in Drug Discovery and Cell-Based Biosensors. *Assay Drug Dev Technol*,

12(4): 207–218.

Fayad, W., Brnjic, S., Berglind, D., Blixt, S., Shoshan, M.C., Berndtsson, M., Olofsson, M.H. & Linder, S. 2009. Restriction of cisplatin induction of acute apoptosis to a subpopulation of cells in a three-dimensional carcinoma culture model. *IJC*, 125(10): 2450–2455.

Foty, R. 2011. A simple hanging drop cell culture protocol for generation of 3D spheroids. *JOVE*, (51): e2720–e2720.

Friedrich, J., Ebner, R. & Kunz-Schughart, L.A. 2009. Experimental anti-tumor therapy in 3-D: Spheroids – old hat or new challenge? *Int J Rad Biol*, 83(11-12): 849–871.

Gaskell, H., Sharma, P., Colley, H.E., Murdoch, C., Williams, D.P. & Webb, S.D. 2016. Characterization of a functional C3A liver spheroid model. *Toxicol. Res.*, 5(4): 1053–1065.

Ghosh, S., Spagnoli, G.C., Martin, I., Ploegert, S., Demougin, P., Heberer, M. & Reschner, A. 2005. Three-dimensional culture of melanoma cells profoundly affects gene expression profile: a high density oligonucleotide array study. *J Cell Physiol*, 204(2): 522–531.

Giaccotto, C., Santerre, R.E. & Vernon, J.A. 2005. Drug Prices and Research and Development Investment Behavior in the Pharmaceutical Industry. *J Law Econ*, 48(1): 195–214.

Goers, L., Freemont, P. & Polizzi, K.M. 2014. Co-culture systems and technologies: taking synthetic biology to the next level. *J Roy Soc interface*, 11(96): 20140065–20140065.

Gomez-Roman, N., Stevenson, K., Gilmour, L., Hamilton, G. & Chalmers, A.J. 2017. A novel 3D human glioblastoma cell culture system for modeling drug and radiation responses. *Neuro-oncology*, 19(2): 229–241.

Gordon, J., Amini, S. & White, M.K. 2013. General overview of neuronal cell culture. *Methods Mol Biol,* 1078(Chapter 1): 1–8.

Griffith, L.G. & Swartz, M.A. 2006. Capturing complex 3D tissue physiology in vitro. *Nat Rev Mol Cell Biol*, 7(3): 211–224.

Guengerich, F.P. 2007. Cytochrome P450 and Chemical Toxicology. *Chem Res Toxicol*, 21(1): 70–83.

Haisler, W.L., Timm, D.M., Gage, J.A., Tseng, H., Killian, T.C. & Souza, G.R. 2013. Three-dimensional cell culturing by magnetic levitation. *Nat Protoc*, 8(10): 1940–1949.

Haouzi, D., Baghdiguian, S., Granier, G., Travo, P., Mangeat, P. & Hibner, U. 2005. Three-dimensional polarization sensitizes hepatocytes to Fas/CD95 apoptotic signalling. *J Cell Sci*, 118(12): 2763–2773.

Hau, H., Khanal, D., Rogers, L., Suchowerska, N., Kumar, R., Sridhar, S., McKenzie, D. & Chrzanowski, W. 2016. Dose enhancement and cytotoxicity of gold nanoparticles in colon cancer cells when irradiated with kilo- and mega-voltage radiation. *Bioeng Transl Med*, 1(1): 94–102.

Härmä, V., Schukov, H.-P., Happonen, A., Ahonen, I., Virtanen, J., Siitari, H., Åkerfelt, M., Lötjönen, J. & Nees, M. 2014. Quantification of Dynamic Morphological Drug Responses in 3D Organotypic Cell Cultures by Automated Image Analysis O. De Wever, ed. *PloS one*, 9(5): e96426–18.

Hickman, J.A., Graeser, R., de Hoogt, R., Vidic, S., Brito, C., Gutekunst, M., van der Kuip, H.IMI

PREDECT Consortium. 2014. Three-dimensional models of cancer for pharmacology and cancer cell biology: capturing tumor complexity in vitro/ex vivo. *Biotechnology*, 9(9): 1115–1128.

Hingorani, P., Zhang, W., Piperdi, S., Pressman, L., Lin, J., Gorlick, R. & Kolb, E.A. 2009. Preclinical activity of palifosfamide lysine (ZIO-201) in pediatric sarcomas including oxazaphosphorine-resistant osteosarcoma. *Canc Chemother Pharmacol*, 64(4): 733–740.

Horman, S.R., Hogan, C., Delos Reyes, K., Lo, F. & Antczak, C. 2015. Challenges and opportunities toward enabling phenotypic screening of complex and 3D cell models. *Future Med Chem*, 7(4): 513–525.

Hughes, J.P., Rees, S., Kalindjian, S.B. & Philpott, K.L. 2011. Principles of early drug discovery. *Br J Pharmacol*, 162(6): 1239–1249.

Ingram, M., Techy, G.B., Saroufeem, R., Yazan, O., Narayan, K.S., Goodwin, T.J. & Spaulding, G.F. 1997. Three-dimensional growth patterns of various human tumor cell lines in simulated microgravity of a NASA bioreactor. *In Vitro Cell Dev Biol Anim*, 33(6): 459–466.

Ivanov, D.P., Parker, T.L., Walker, D.A., Alexander, C., Ashford, M.B., Gellert, P.R. & Garnett, M.C. 2014. Multiplexing spheroid volume, resazurin and acid phosphatase viability assays for high-throughput screening of tumour spheroids and stem cell neurospheres. M. A. Mancini, ed. *PloS one*, 9(8): e103817.

Jaganathan, H., Gage, J., Leonard, F., Srinivasan, S., Souza, G.R., Dave, B. & Godin, B. 2014. Three-Dimensional In Vitro Co-Culture Model of Breast Tumor using Magnetic Levitation. *Nat Publ Gr*, 4: 6468–9.

Jang, M., Neuzil, P., Volk, T., Manz, A. & Kleber, A. 2015. On-chip three-dimensional cell culture in phaseguides improves hepatocyte functions in vitro. *Biomicrofluidics*, 9(3): 034113.

Jeon, O., Marks, R., Wolfson, D. & Alsberg, E. 2016. Dual-crosslinked hydrogel microwell system for formation and culture of multicellular human adipose tissue-derived stem cell spheroids. *J Mater Chem*, 4(20): 3526–3533.

Jeong, S.-Y., Lee, J.-H., Shin, Y., Chung, S. & Kuh, H.-J. 2016. Co-Culture of Tumor Spheroids and Fibroblasts in a Collagen Matrix-Incorporated Microfluidic Chip Mimics Reciprocal Activation in Solid Tumor Microenvironment J. W. Lee, ed. *PloS one*, 11(7): e0159013–17.

Kalluri, R. 2016. The biology and function of fibroblasts in cancer. *Nat Rev Cancer*, 16(9): 582–598.

Katt, M.E., Placone, A.L., Wong, A.D., Xu, Z.S. & Searson, P.C. 2016. In Vitro Tumor Models: Advantages, Disadvantages, Variables, and Selecting the Right Platform. *Front Bioeng Biotechnol*, 4(Suppl. 1): 12.

Kelm, J.M., Timmins, N.E., Brown, C.J., Fussenegger, M. & Nielsen, L.K. 2003. Method for generation of homogeneous multicellular tumor spheroids applicable to a wide variety of cell types. *Biotechnol Bioeng*, 83(2): 173–180.

Kim, S.-A., Lee, E.K. & Kuh, H.-J. 2015. Co-culture of 3D tumor spheroids with fibroblasts as a model for epithelial-mesenchymal transition in vitro. *Exp Cell Res*, 335(2): 187–196.

Kimlin, L.C., Casagrande, G. & Virador, V.M. 2013. In vitro three-dimensional (3D) models in cancer research: an update. *Mol Carcinogen*, 52(3): 167–182.

Knight, A. 2008. Systematic reviews of animal experiments demonstrate poor contributions toward human healthcare. *Rev Rec Clin Trials. 3(2):89-96.*

Kobet, R.A., Pan, X., Zhang, B., Pak, S.C., Asch, A.S. & Lee, M.-H. 2014. Caenorhabditis elegans: A Model System for Anti-Cancer Drug Discovery and Therapeutic Target Identification. *Biomol Therap*, 22(5): 371–383.

Lakehal, F., Wendum, D., Barbu, V., Becquemont, L., Poupon, R., Balladur, P., Hannoun, L., Ballet, F., Beaune, P.H. & Housset, C. 1999. Phase I and phase II drug-metabolizing enzymes are expressed and heterogeneously distributed in the biliary epithelium. *Hepatology*, 30(6): 1498–1506.

Lee, G.Y., Kenny, P.A., Lee, E.H. & Bissell, M.J. 2007. Three-dimensional culture models of normal and malignant breast epithelial cells. *Nat Methods*, 4(4): 359–365.

Li, L. & Lu, Y. 2011. Optimizing a 3D Culture System to Study the Interaction between Epithelial Breast Cancer and Its Surrounding Fibroblasts. *J Cancer*, 2: 458–466.

Lin, R.Z. & Chang, H.Y. 2008. Recent advances in three-dimensional multicellular spheroid culture for biomedical research. *Biotechnol J*, 3(9-10): 1172–1184.

Loessner, D., Stok, K.S., Lutolf, M.P., Hutmacher, D.W., Clements, J.A. & Rizzi, S.C. 2010. Bioengineered 3D platform to explore cell-ECM interactions and drug resistance of epithelial ovarian cancer cells. *Biomaterials*, 31(32): 8494–8506.

Longley, D.B., Harkin, D.P. & Johnston, P.G. 2003. 5-Fluorouracil: mechanisms of action and clinical strategies. *Nat rev Cancer*, 3(5): 330–338.

Luca, A.C., Mersch, S., Deenen, R., Schmidt, S., Messner, I., Schäfer, K.-L., Baldus, S.E., Huckenbeck, W., Piekorz, R.P., Knoefel, W.T., Krieg, A. & Stoecklein, N.H. 2013. Impact of the 3D microenvironment on phenotype, gene expression, and EGFR inhibition of colorectal cancer cell lines. N. Cordes, ed. *PloS one*, 8(3): e59689.

MacNeil, S. 2007. Progress and opportunities for tissue-engineered skin. *Nature*, 445(7130): 874–880.

Magdeldin, T., López-Dávila, V., Villemant, C., Cameron, G., Drake, R., Cheema, U. & Loizidou, M. 2014. The efficacy of cetuximab in a tissue-engineered three-dimensional in vitro model of colorectal cancer. *J Tissue Eng*, 5: 2041731414544183.

Mani, S.A., Guo, W., Liao, M.-J., Eaton, E.N., Ayyanan, A., Zhou, A.Y., Brooks, M., Reinhard, F., Zhang, C.C., Shipitsin, M., Campbell, L.L., Polyak, K., Brisken, C., Yang, J. & Weinberg, R.A. 2008. The Epithelial-Mesenchymal Transition Generates Cells with Properties of Stem Cells. *Cell*, 133(4): 704–715.

Maria, O.M., Zeitouni, A., Gologan, O. & Tran, S.D. 2011. Matrigel improves functional properties of primary human salivary gland cells. *Tissue Eng. Part A*, 17(9-10): 1229–1238.

Mehta, G., Hsiao, A.Y., Ingram, M., Luker, G.D. & Takayama, S. 2012. Opportunities and challenges for use of tumor spheroids as models to test drug delivery and efficacy. *J Control Release*, 164(2): 192–204.

Mellor, H.R., Snelling, S., Hall, M.D., Modok, S., Jaffar, M., Hambley, T.W. & Callaghan, R. 2005. The influence of tumour microenvironmental factors on the efficacy of cisplatin and novel

platinum(IV) complexes. *Biochem Pharmacol*, 70(8): 1137–1146.

Messner, S., Agarkova, I., Moritz, W. & Kelm, J.M. 2013. Multi-cell type human liver microtissues for hepatotoxicity testing. *Arch Toxicol*, 87(1): 209–213.

Miki, Y., Ono, K., Hata, S., Suzuki, T., Kumamoto, H. & Sasano, H. 2012. The advantages of co-culture over mono cell culture in simulating in vivo environment. J Steroid Biochem Mol Biol, 131(3-5): 68–75.

Minchinton, A.I. & Tannock, I.F. 2006. Drug penetration in solid tumours. *Nat Rev Cancer*, 6(8): 583–592.

Mosadegh, B., Lockett, M.R., Minn, K.T., Simon, K.A., Gilbert, K., Hillier, S., Newsome, D., Li, H., Hall, A.B., Boucher, D.M., Eustace, B.K. & Whitesides, G.M. 2015. A paper-based invasion assay: Assessing chemotaxis of cancer cells in gradients of oxygen. *Biomaterials*, 52: 262–271.

Mueller-Klieser, W. 1997. Three-dimensional cell cultures: from molecular mechanisms to clinical applications. *Am J Physiol*, 273(4 Pt 1): C1109–23.

Ocana, A., Pandiella, A., Siu, L.L. & Tannock, I.F. 2010. Preclinical development of molecular-targeted agents for cancer. *Nat Rev Clin Oncol*, 8(4): 200–209.

Olive, P.L. & Durand, R.E. 1994. Drug and radiation resistance in spheroids: cell contact and kinetics. *Cancer Metastasis Rev*, 13(2): 121–138.

Olsavsky, K.M., Page, J.L., Johnson, M.C., Zarbl, H., Strom, S.C. & Omiecinski, C.J. 2007. Gene expression profiling and differentiation assessment in primary human hepatocyte cultures, established hepatoma cell lines, and human liver tissues. *Toxicol Appl Pharm*, 222(1): 42–56.

Pàez-Ribes, M., Allen, E., Hudock, J., Takeda, T., Okuyama, H., Viñals, F., Inoue, M., Bergers, G., Hanahan, D. & Casanovas, O. 2009. Antiangiogenic Therapy Elicits Malignant Progression of Tumors to Increased Local Invasion and Distant Metastasis. *Cancer cell*, 15(3): 220–231.

Ramaiahgari, S.C., Braver, den, M.W., Herpers, B., Terpstra, V., Commandeur, J.N.M., van de Water, B. & Price, L.S. 2014. A 3D in vitro model of differentiated HepG2 cell spheroids with improved liver-like properties for repeated dose high-throughput toxicity studies. *Arch Toxicol*: 1–13.

Ravi, M., Paramesh, V., Kaviya, S.R., Anuradha, E. & Solomon, F.D.P. 2015. 3D cell culture systems: advantages and applications. *J Cell Physiol*, 230(1): 16–26.

Rotem, A., Janzer, A., Izar, B., Ji, Z., Doench, J.G., Garraway, L.A. & Struhl, K. 2015. Alternative to the soft-agar assay that permits high-throughput drug and genetic screens for cellular transformation. *PNAS*, 112(18): 5708–5713.

Sakai, S., Ito, S. & Kawakami, K. 2010. Calcium alginate microcapsules with spherical liquid cores templated by gelatin microparticles for mass production of multicellular spheroids. *Acta Biomater*, 6(8): 3132–3137.

Santini, M.T. & Rainaldi, G. 1999. Three-dimensional spheroid model in tumor biology. *Pathobiology,*67(3): 148–157.

Sawant, S., Dongre, H., Singh, A.K., Joshi, S., Costea, D.E., Mahadik, S., Ahire, C., Makani, V., Dange, P., Sharma, S., Chaukar, D. & Vaidya, M. 2016. Establishment of 3D Co-Culture Models from

Different Stages of Human Tongue Tumorigenesis: Utility in Understanding Neoplastic Progression C. Murdoch, ed. *PloS one*, 11(8): e0160615–20.

Strese, S., Fryknäs, M., Larsson, R. & Gullbo, J. 2013. Effects of hypoxia on human cancer cell line chemosensitivity. *BMC cancer*, 13(1): 331.

Takahashi, Y., Hori, Y., Yamamoto, T., Urashima, T., Ohara, Y. & Tanaka, H. 2015. Three-dimensional (3D) spheroid cultures improve the metabolic gene expression profiles of HepaRG cells. *Biosci Rep*: 1–7.

Thoma, C.R., Zimmermann, M., Agarkova, I., Kelm, J.M. & Krek, W. 2014. 3D cell culture systems modeling tumor growth determinants in cancer target discovery. *Adv Drug Deliv Rev*, 69-70: 29–41.

Tibbitt, M.W. & Anseth, K.S. 2009. Hydrogels as extracellular matrix mimics for 3D cell culture. *Biotechnol Bioeng*, 103(4): 655–663.

Torre, L.A., Bray, F., Siegel, R.L., Ferlay, J., Lortet-Tieulent, J. & Jemal, A. 2015. Global cancer statistics, 2012. *CA Cancer J Clin*, 65(2): 87–108.

Tung, Y.-C., Hsiao, A.Y., Allen, S.G., Torisawa, Y.-S., Ho, M. & Takayama, S. 2011. High-throughput 3D spheroid culture and drug testing using a 384 hanging drop array. *The Analyst*, 136(3): 473–478.

van Duinen, V., Trietsch, S.J., Joore, J., Vulto, P. & Hankemeier, T. 2015. Microfluidic 3D cell culture: from tools to tissue models. *Curr Opin Biotechnol*, 35: 118–126.

Verbridge, S.S., Chakrabarti, A., DelNero, P., Kwee, B., Varner, J.D., Stroock, A.D. & Fischbach, C. 2013. Physicochemical regulation of endothelial sprouting in a 3D microfluidic angiogenesis model. *J Biomed Mater Res A*, 101(10): 2948–2956.

Vinci, M., Gowan, S., Boxall, F., Patterson, L., Zimmermann, M., Court, W., Lomas, C., Mendiola, M., Hardisson, D. & Eccles, S.A. 2012. Advances in establishment and analysis of three-dimensional tumor spheroid-based functional assays for target validation and drug evaluation. *BMC biol*, 10(1): 10-29.

Walenta, S., Dötsch, J., Bourrat-Flöck, B. & Mueller-Klieser, W. 1990. Size-dependent oxygenation and energy status in multicellular tumor spheroids. *Adv Exp Med Biol*, 277: 889–893.

Walrath, J.C., Hawes, J.J., Van Dyke, T. & Reilly, K.M. 2010. Genetically engineered mouse models in cancer research. *Adv Cancer Res*.

Waring, M.J., Arrowsmith, J., Leach, A.R., Leeson, P.D., Mandrell, S., Owen, R.M., Pairaudeau, G., Pennie, W.D., Pickett, S.D., Wang, J., Wallace, O. & Weir, A. 2015. An analysis of the attrition of drug candidates from four major pharmaceutical companies. *Nat Rev Drug Discov*, 14(7): 475–486.

Wartenberg, M., Ling, F.C., Müschen, M., Klein, F., Acker, H., Gassmann, M., Petrat, K., Pütz, V., Hescheler, J. & Sauer, H. 2003. Regulation of the multidrug resistance transporter P-glycoprotein in multicellular tumor spheroids by hypoxia-inducible factor (HIF-1) and reactive oxygen species. *FASEB J,* 17(3): 503–505.

Weaver, V.M., Petersen, O.W., Wang, F., Larabell, C.A., Briand, P., Damsky, C. & Bissell, M.J. 1997. Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in

vivo by integrin blocking antibodies. *J Cell Biol*, 137(1): 231–245.

Weiswald, L.-B., Bellet, D. & Dangles-Marie, V. 2015. Spherical Cancer Models in Tumor Biology. *NEO*, 17(1): 1–15.

Wiśniewski, J.R., Vildhede, A., Norén, A. & Artursson, P. 2016. In-depth quantitative analysis and comparison of the human hepatocyte and hepatoma cell line HepG2 proteomes. *J Proteomics*, 136(C): 234–247.

Yamada, K.M. & Cukierman, E. 2007. Modeling Tissue Morphogenesis and Cancer in 3D. *Cell*, 130(4): 601–610.

Yoshii, Y., Waki, A., Yoshida, K., Kakezuka, A., Kobayashi, M., Namiki, H., Kuroda, Y., Kiyono, Y., Yoshii, H., Furukawa, T., Asai, T., Okazawa, H., Gelovani, J.G. & Fujibayashi, Y. 2011. The use of nanoimprinted scaffolds as 3D culture models to facilitate spontaneous tumor cell migration and well-regulated spheroid formation. *Biomaterials*, 32(26): 6052–6058.

Zanoni, M., Piccinini, F., Arienti, C., Zamagni, A., Santi, S., Polico, R., Bevilacqua, A. & Tesei, A. 2015. 3D tumor spheroid models for in vitro therapeutic screening: a systematic approach to enhance the biological relevance of data obtained. *Nat Publ Gr*, 6: 1–11.

Zhang, S., Gelain, F. & Zhao, X. 2005. Designer self-assembling peptide nanofiber scaffolds for 3D tissue cell cultures. *Semin Cancer Biol*, 15(5): 413–420.

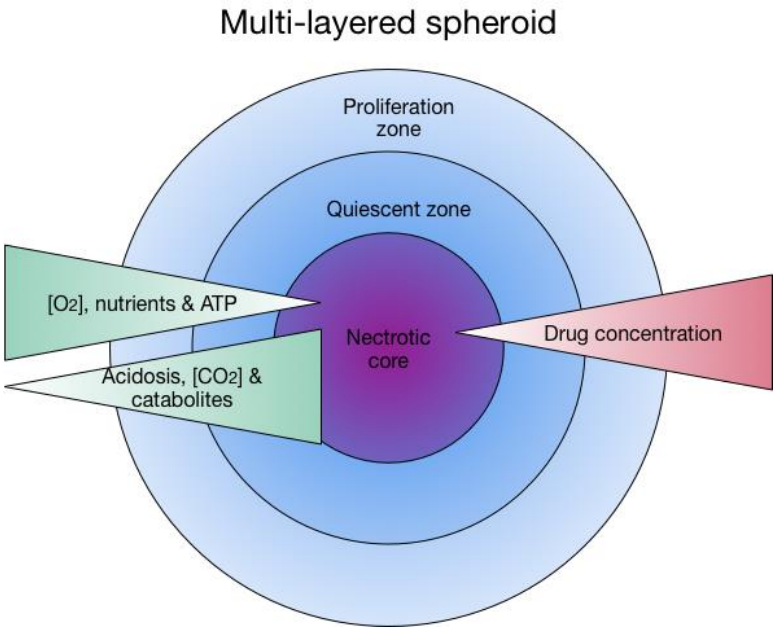

Fig.1. Schematic representation of multi-layered spherical geometry. Proliferation is higher on the outside
due to high levels of oxygen and nutrients. The availability of nutrients and oxygen decreases towards the
center of the spheroid resulting in growth arrest or cell necrosis (adapted from Lin and Chang 2008).

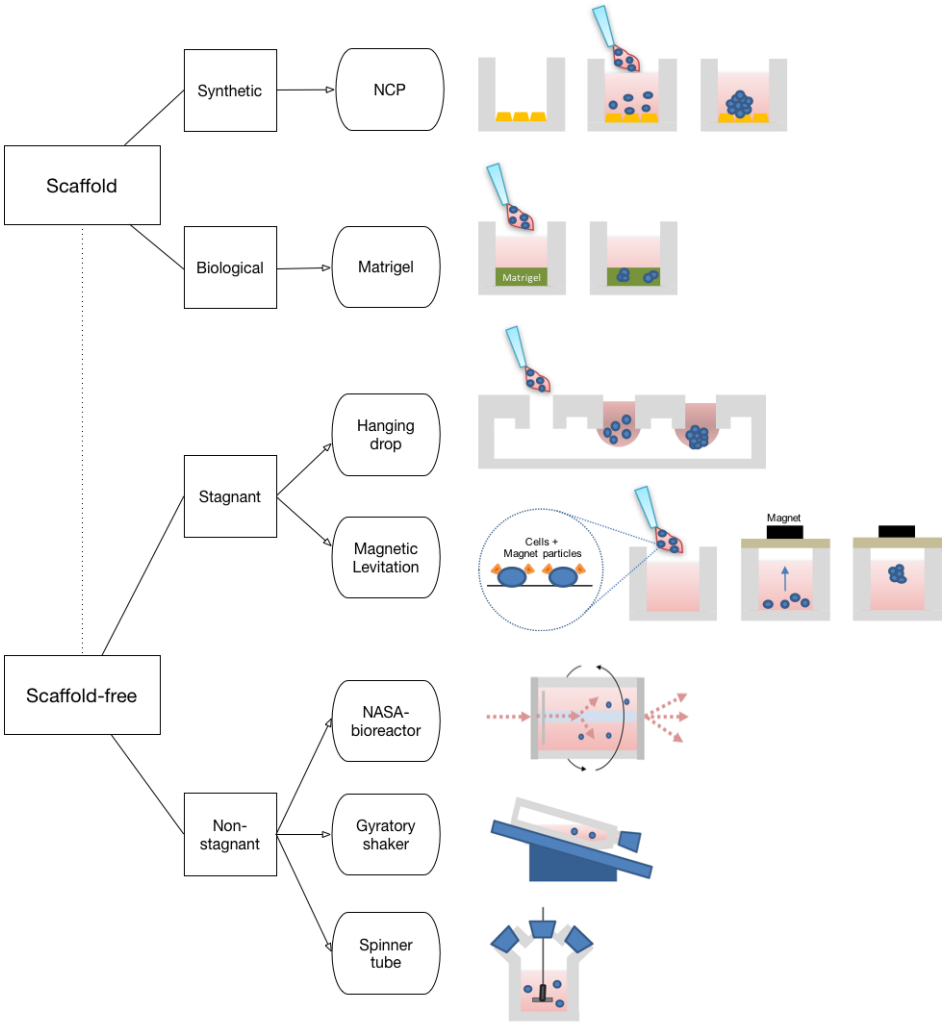

Fig.2. Schematic overview of the different 3D cell culturing systems (adapted from Friedrich et al. 2009 as
well as Li and Lu 2011).