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https://doi.org/10.22239/2317-269x.01047

Three-dimensional cell culture: nearing the gap between *in vitro* and *in vivo* models

Modelos tridimensionais de cultura de células: aproximando o *in vitro* do *in vivo*

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ABSTRACT

Introduction: Biotechnological advances in association with the pressure to substitute animal experimentation impelled the development of *in vitro* models that are more physiological and predictive of *in vivo* response. **Objective:** To discuss advantages and limitations of three-dimensional (3D) cell culture models. **Method:** Review of the scientific literature at PubMed using the keywords "3D culture", spheroid, organoid, "organotypic culture", "alternative model", microfluidic, organ-on-a-chip and biotechnology, individually and in different combinations. The search period was from 1971 to 2017. **Results:** Traditional monolayer cell culture assays, although extensively used, do not reproduce the cell-cell and cell-extracellular matrix interactions that create physical and chemical gradients and that control cell functions, such as survival, proliferation, differentiation, migration, and protein and gene expression. 3D cell culture models are able to mimic more physiological microenvironment. The number of manuscripts published in this period reflects the scientific interest in the field. **Conclusions:** Although 3D models have unequivocally contributed to the bioengineering, morphogenesis, oncology, and toxicology fields, many challenges remain. The high cost of some of these models, to reproduce the mechanical spatiotemporal features of the tissues, as wells as the lack of standard protocols should be taken into account. Here we discuss the advantages and limitations of some 3D cell culture models.

KEYWORDS: 3D Culture; Alternative Model; Multicellular Spheroid; Organotypic Culture; Organoid

RESUMO

Introdução: Os avanços biotecnológicos em associação com a pressão para substituir a experimentação animal impulsionam o desenvolvimento de modelos *in vitro* mais fisiológicos e preditivos da resposta *in vivo*. **Objetivo:** Discutir vantagens e limitações de modelos tridimensionais (3D) de cultura de células. **Método:** Revisão da literatura na base PubMed utilizando os termos "*3D culture*", *spheroid*, *organoid*, "*organotypic culture*", "*alternative model*", *microfluidic*, *organ-on-a-chip* e *biotechnology*, individualmente e em diferentes combinações. A pesquisa abrangeu o período de 1971 a 2017. **Resultados:** Ensaios tradicionais de cultura em monocamada, embora sejam amplamente utilizados, não reproduzem as interações célula-célula e célula-matriz extracelular, que criam gradientes físicos e químicos e controlam funções celulares, como sobrevivência, proliferação, diferenciação, migração e expressão de genes e proteínas. Modelos 3D de cultura de células são capazes de mimetizar um microambiente mais fisiológico. O número de publicações no período estudado reflete o crescente interesse científico no tema. **Conclusões:** Embora os modelos 3D tenham inequivocamente contribuído para as áreas de bioengenharia, morfogênese, oncologia e toxicologia, muitos desafios permanecem. O custo elevado de alguns destes modelos, reproduzir as características mecânicas, espaciais e temporais dos tecidos, assim como a necessidade de desenvolver protocolos padronizados devem ser considerados.

PALAVRAS-CHAVE: Cultura 3D; Modelo Alternativo; Esferoide Multicelular; Cultura Organotípica; Organoide

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Received: Sep 30, 2017
Approved: Jan 23, 2018

INTRODUCTION

From the last decade of the twentieth century until now, the technological progress in the area of biology and health has been enormous. Tools and methodologies have been developed to enable the handling of DNA and RNA molecules and the *in vitro* development of tissues and organs with similar characteristics to those observed *in vivo*. The ability to sequence the genome quickly and efficiently increased significantly from 1995, the year of the first scientific publication on the subject, to 2001, when the first results of the human genome project were published[1,2]. At the same time, the knowledge acquired in recent decades on stem cells derived from the inner layer of blastocysts (embryonic stem cells), adult tissues and induced pluripotency (iPSC – induced Pluripotent Stem Cell) has enabled substantial progress in the area of Regenerative Medicine, which includes cell therapy and bioengineering[3,4,5,6,7,8], with 6,205 clinical trials using stem cells recorded to date (Source: https://clinicaltrials.gov). That is, there has been a continuous expansion of the area of Biotechnology, defined as "any technological application that uses biological systems, living organisms, or their derivatives, to manufacture or modify products or processes for specific use", as defined by art. 2 of the text approved by the Convention on Biological Diversity, signed at the United Nations Conference on Environment and Development (Rio de Janeiro, June 5 to 14, 1992) and approved by Legislative Decree n. 2 of 1994. Undoubtedly, biotechnology entails enormous possibilities, such as the creation of synthetic organisms[9,10] and changes in how we deal with diseases caused by genetic mutation[11,12]. However, it is unquestionable that its likely progress in the coming years creates ethical and regulatory challenges, which have already been debated in developed countries[11,13,14].

In Brazil, the debate moves more slowly towards the approval of cellular therapies and the marketing of engineered tissues from human cells. The controversy over marketing rests on § 4 of art. 199 of the Brazilian Federal Constitution which, despite of approving the "removal of human organs, tissues and substances", including the "collection, processing and transfusion of blood and its products", limits these practices to "transplantation, research and treatment" interposing "any type of marketing"[15]. The Brazilian legislation still has provisions such as Law n. 9.434 of February 4, 1997 and Law n. 11.105 of March 24, 2005 (Biosafety Law), which provide, respectively, for the free disposal of organs, tissues or parts of the body and the safety and inspection regulations for activities involving genetically modified organisms (GMOs)[16,17]. The Brazilian National Agency of Sanitary Surveillance (Anvisa) has made some discrete progress toward the development of more significant guidelines in the area. This is the case of the Resolution of the Board of Directors (RDC) n. 9 of March 14, 2011, which "provides for the operation of Cellular Technology Centers for the purpose of clinical research and therapy"[18]. This question, although urgent and necessary, will not be addressed more broadly in this study because it requires a specific forum.

New biotechnology products in the health area require platforms or models to evaluate their efficacy and safety prior to their application in clinical trials. In general, preclinical tests involve *in vitro* models with monolayer cell culture, mostly from immortalized and commercially available cell lineages, and *in vivo* models. *In vivo* tests, which use laboratory animals, are often unsuitable due to species-specific differences. In addition to that, as a consequence of the concern about the use of animals as experimental models, the policy of replacement, reduction and refinement, called the 3Rs policy, was introduced in the 1950s. In the United Kingdom, the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs - www.nc3rs.org.uk) was created with the mission of finding innovative solutions to achieve the objectives of the 3Rs policy. This also promoted the development of alternative methods to animal use[19,20]. The biotechnological progress itself is a driving force to the development of reproducible alternative models that are closer to human biology and consequently have greater predictive power.

The objective was to present several types of three-dimensional culturing models from a historical perspective and to critically discuss advantages and limitations of these as to their predictive power and reproducibility for implantation as an alternative to the use of experimental animals. In view of the vast literature, we did not intend to exhaust the topic and, thus, additional information was indicated.

METHOD

Here we present a narrative review of the literature on three-dimensional culture models, without exhausting the topic, given the vastness of related articles, which reflects today's great scientific interest in the area. The literature review was performed through the PubMed database (https://www.ncbi.nlm.nih.gov/pubmed), using the following expressions: "*3D culture*", *spheroid*, *organoid*, "*organotypic culture*", "*alternative model*", *microfluidic*, *organ-on-a-chip* and *biotechnology*, individually and in many combinations like: (a) "*3D culture*" OR *spheroid*; (b) "*3D culture*" AND "*alternative model*" and (c) "*3D culture*" AND "*biotechnology*". The research covered the period from 1971 to 2017 and the articles were selected for their historical and scientific importance, taking into account their availability on the Capes portal (www.periodicos.capes.gov.br) or free access. Given the number of publications on the topic (total of 5,497 in the period, using only the keywords spheroid or "*3D culture*"), review articles were selected as a source of supplementary consultation. Additional information on biotechnology was obtained on the page of the Brazilian government with a collection of federal laws and the 1988 Constitution (http://www4.planalto.gov.br/legislacao). In addition, the NC3Rs website (www.nc3rs.org.uk) was consulted for further information on the policy of reducing, replacing and refining the use of experimental animals.

RESULTS AND DISCUSSION

Two-dimensional and three-dimensional animal cell culture models: history

Animal cell cultures were introduced in the early twentieth century as a method of study. After the isolation of the first lineage of human tumor cells in the 1950s, HeLa cells were increasingly used[21,22]. Although this technique has allowed undeniable progress in the field of cell biology and is still widely used, from the late twentieth and early twenty-first century the limitations of the model have become more evident, especially with regard to the testing of new drugs. In a traditional culture, generally referred to as two-dimensional (2D) culture, the cells tend to form a monolayer adhered to a modified polystyrene surface to promote adhesion, which is obviously a substrate not found *in vivo* and which induces an artificial polarization of the cells, which is not observed when they are in the tissues. *In vivo*, the cells interact with each other through adhesion molecules, mainly of the cadherin family, and through junctional complexes (desmosomes, intimate or occlusive junction and adhesion junction) that bind, in their cytoplasmic portion, to cytoskeleton, via adapter molecules[23,24,25,26]. Furthermore, depending on their type, the cells are: (a) immersed in an extracellular matrix of varied composition, which includes several types of collagen, elastic fibers, various glycoproteins, such as fibronectin, laminin and vitronectin, as well as proteoglycans and glycosaminoglycans or b) supported on a basal lamina formed predominantly by laminin, in addition to collagen IV and proteoglycans. The cells bind to the extracellular matrix via molecules mainly of the integrin type, which also interact, via adapter molecules, to the cytoskeleton [27,28,29]. These cell-cell and extracellular matrix-cell interactions create mechanical forces that spatially organize both the extracellular matrix and the cellular components (cytoskeleton and organelles), modulating various cellular properties, such as shape, differentiation, and migration[26,30,31,32,33,34,35,36]. Associated with chemical gradients generated by diffusion of fluids, O_2 and cellular metabolites[37,38,39,40], and by the association of growth factors and chemokines with extracellular matrix molecules[27,41], these interactions form specific microenvironments or niches that regulate tissue homeostasis[42,43,44,45]. Both the complex cellular interactions and the physical and chemical gradients observed in the tissues are not reproduced in monolayer cultures on plastic[33,40,46,47,48,49,50,51,52,53,54]. In addition to that, possibly as a result of their adaptation to monolayer culture conditions, the cells modify the pattern of gene expression[49,51,54,55,56]. Therefore, it is not surprising that, depending on the study objectives, the results observed with these *in vitro* models are very often not reproduced *in vivo*. In fact, the instigating results of the group of Dr. Mina Bissell[57,58], obtained from cell culture in a three-dimensional (3D) system, changed the landscape in the area of oncology, deserving an editorial on *Nature* called "Goodbye, flat biology?"[59] and the launch by the National Cancer Institute (NCI) in the United States of a research program, started in October 2003, with an annual budget of 400 million USD for 3D culture[60]. This has stimulated the development of *in vitro* systems that seek to mimic the physiology and histology of human organs and tissues, which is reflected by the increase in the number of scientific publications on the subject since then[51]. A survey on the PubMed portal using the terms "*3D culture*" OR "*spheroid*" resulted in 5,497 articles published in the period of 1971 to 2017. The analysis over time indicated a large increase in the number of articles published since the beginning of the twentieth century. From 1971 to 2000, the average was 37 articles a year, increasing to 261 articles a year after 2001. Only in 2017 a total of 936 articles were published, which demonstrates the interest in the topic and the impact of the results obtained with *in vitro* three-dimensional models.

Types of 3D culture models

Various culture systems with an approach where a more physiological, complex and three-dimensional organization of cells occur have been developed since the earliest reports in 1971. Techniques referred to as 3D culture, organotypic cultures or organoid cultures include systems where cells are cultured in three-dimensional molds of varying composition, models in which cells or organ fragments are mechanically sustained (Figure 1) and suspended aggregate models, called spheroids because of their rounded appearance (Figure 2). More recently, microproduced microfluidics culture systems and the so-called organ-on-a-chip systems have been introduced.

Culture in molds of biopolymers, ceramics or metal

The models that use molds seek to create an environment that mimics the extracellular matrix and, consequently, regulates the spatial organization of cells, their migration, proliferation and differentiation. A wide variety of substances, such as extracellular matrix molecules, natural biopolymers, synthetic or hybrid polymers, ceramics and metals, have been used as molds[49,51,53,61,62,63,64]. The biomaterials vary in their rigidity, porosity and biodegradable potential and the choice of the material depends on both the cell type and the application of the study. Growth factors can be incorporated, favoring the proliferation and differentiation of the cells cultured in these molds and making them more physiological, that is, more similar to the extracellular matrix of the tissues. The variety of biomaterials and their applications are the subject of specific reviews[49,51,53,63,64].

Extracellular matrix or biopolymer systems were initially proposed with the culturing of primary cells (obtained by *ex vivo* tissue dissociation) or established lineage, in suspension or as aggregates, on extracellular matrix substrate, which does not properly constitute a 3D culture (Figure 1A). Nevertheless, it was verified that this type of culture favored the proliferation and the differentiation of the cells, allowing them to associate in a more physiological way, mimicking their *in vivo* organization. These studies contributed to the understanding of the impact of extracellular cell-matrix interactions on cell properties and how these were modified depending on the type of substrate[49,63,64,65,66,68].

In an attempt to reconstitute a more physiological environment, the cells were encapsulated in the polymerized matrix, which forms a gel (Figure 1B). In this three-dimensional environment,

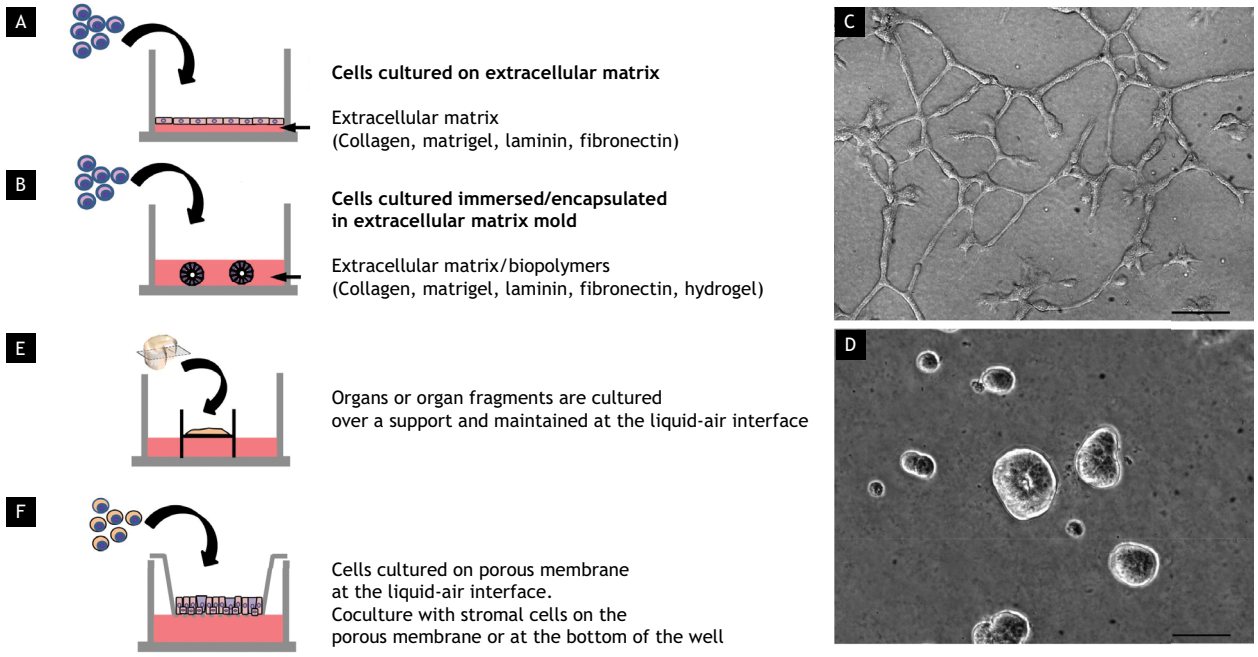

Figure 1. Cell culture models and organotypic cultures. (A) Biopolymer culture model. The cells are distributed in culture flasks whose surface has been coated with extracellular matrix molecules, such as collagen I and matrigel. (B) Model of culture in mold of natural or synthetic biopolymer. The cells are encapsulated in biopolymer gel, which enables their more physiological (C-D) three-dimensional organization. MDA-MB-231 (C) and luminal T-47D (D) human lineages of basal breast tumor were encapsulated in matrigel (3D culture), forming, respectively, structures that were similar to branched ducts (C) or acini (D). Phase contrast. Bars = 100 µm (C) and 50 µm (D). (E-F) Culture on mechanical support. Organs or fragments of organs (E) and cells (F) are cultured in an insert at the liquid-air interface.

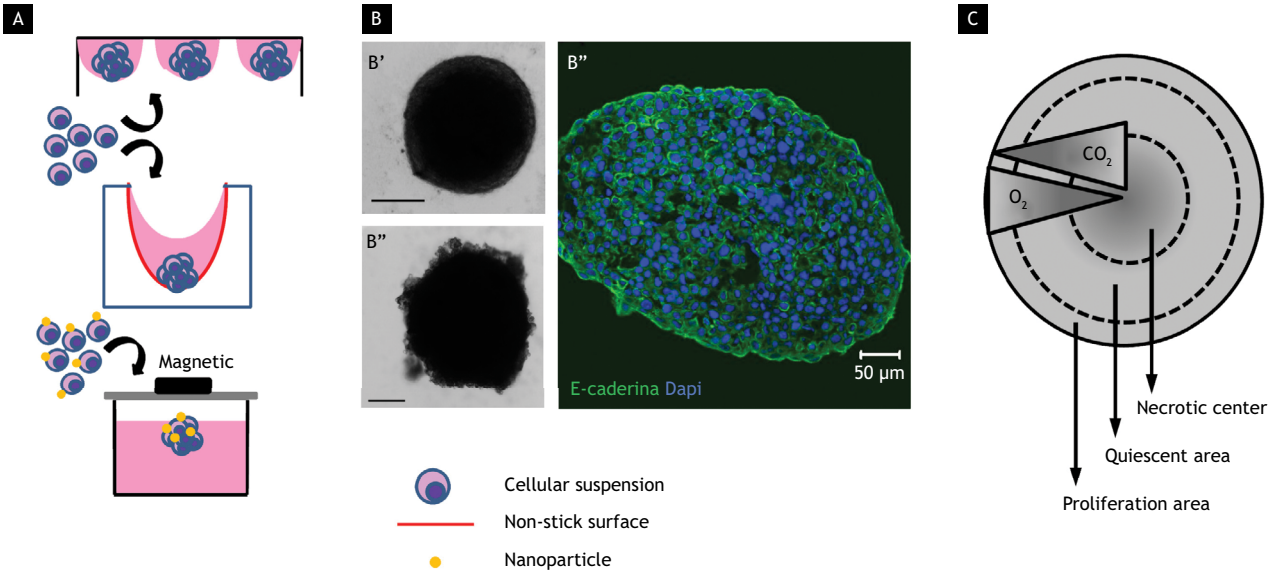

Figure 2. Multicellular spheroid type 3D culture model. (A) Cell suspension cultured using the hanging drop technique (above) in culture flask with modified surface to prevent adhesion (medium) and using the magnetic levitation technique, in which the cells are incubated with nanoparticles and exposed to a magnetic field. (B) Morphology of human fibroblast spheroids (B') and human lineages of luminal breast tumor MCF-7 (B'') and basal breast tumor MDA-MB-231 (B'''). Note the irregular surface of the MDA-MB-231 spheroid compared to that of the fibroblast and the expression of the E-cadherin (green) adhesion molecule in MCF-7 cells. Cores stained with DAPI (in blue). Phase contrast (B' and B''') and confocal microscopy (B''). Bars = 50µm. (C) Spheroid scheme showing O_2 and CO_2 gradient from the center to the periphery and the regions of proliferation, quiescence and cell death that can be observed are indicated.

in contact with extracellular matrix molecules, different cell types (tumor and fibroblasts, among others) were able to migrate in a very similar way to that observed *in vivo*, which enabled the researchers to investigate the details of the cell-matrix interaction during migration[69,70]. Moreover, these models allow cells to organize themselves in complex ways. For example, cells derived

from mammary glands form structures similar to branched ducts or acini[71,72] (Figure 1C-D). That is, in a 3D environment, cells organize spontaneously forming complex histological structures, which resemble those observed in the organs from which they derive. For this reason, these structures have been called organoids (*oides* means similar in Latin). However, some groups of scientists restrict the term organoid to 3D models initiated with stem or progenitor cells, which proliferate and differentiate, generating a progeny that forms histological structures similar to those of the organs of origin[63,73,74]. Intestine organoids (mini-guts) were described in 2009 from cells with stem cell potential, isolated from small intestine crypts, which were cultivated in 3D model of matrigel. The system allowed the proliferation and differentiation of these cells that originated the other populations of the intestinal epithelium (enterocytes, goblet cells and Paneth cells) and formed structures similar to intestinal crypts and villi[75,76]. That is, the model was able to reveal the differentiation potential of the target cells, confirming their identity as intestinal stem cells. Brain organoids (mini-brains) were developed from an adaptation of the neuroectoderm induction model into embryonic bodies formed by embryonic stem cells[77]. In addition to understanding the morphogenesis of the nervous tissue, these organoids proved to be important tools in the description of the pathological mechanism of the Zika virus and its possible implication in the development of microcephaly[78]. The examples above show the potential applications of this culture system in understanding tissue morphogenesis and disease modeling, and it is therefore not surprising that a growing list of other models, such as liver, retina, pituitary, lung, can be found in scientific literature[63,73,74,79].

Mechanically supported 3D culture models

Organ cultures and tissue fragments were introduced in the mid-twentieth century. Since the conditions of nutrient diffusion and gas exchange are not ideal if tissues are immersed in liquid medium, the culture strategies were oriented to favor these processes. Thus, organ cultures utilize a support that allows the tissues to be in a liquid-air interface (Figure 1E). Initially, the supports were mounted with a microporous filter applied to a metal grid, collagen gel or sponge, but recently, commercially available porous membrane inserts are the most commonly used supports[21,63,80,81]. This type of culture maintains the histological characteristics of the tissues and has brought about advances in several areas of knowledge. Among the various organ culture types, the fetal thymus organ culture (FTOC) was one of the most widely used, having contributed significantly to the understanding of the stages and mechanisms of differentiation of T lymphocytes[80,81,82,83].

This type of mechanically supported model was more recently adapted for culturing cells that traditionally are in contact with air *in vivo*, like keratinocytes and respiratory epithelium (Figure 1F). Cells are cultured on the porous membrane of the insert and exposed to the liquid-air interface. The surface of the membrane can be covered by extracellular matrix and, furthermore, this model allows co-culturing with fibroblasts, which, immersed in the collagen matrix, mimic the underlying stroma. When cultured in a liquid medium, keratinocytes derived from the epidermis form a monolayer, but when exposed to a liquid-air interface, they stratify and differentiate into a keratinized layer[63,84]. Similarly, the conducting airway cells cultured in this system are more physiologically organized, reproducing the morphology of the respiratory epithelium, that is, a cylindrical ciliated pseudostratified epithelium, where goblet cells can be observed[85,86,87,88]. The potential of these culture systems is evident, not only for understanding morphogenesis mechanisms and biological properties of epithelia, but above all as alternative models for testing new drugs and cytotoxicity. In fact, epidermal models like Episkin (L'Oréal and Shanghai Episkin Biotechnology Ltd.) and respiratory epithelium such as EpiAirway™ (MatTek Corporation) and MucilAir™ (Epithelix) are currently commercially available.

3D cell aggregates: multicellular spheroid

The 3D multicellular spheroid culture model was initially developed as cell aggregates for the study of developmental biology in the 1940-1950 decades[89,90,91]. In the 1970s, Sutherland et al.[92] boosted research in the area of oncology by proposing the model for the systematic study of tumor response to radiation therapy and drugs. The multicellular spheroid model is based on the ability of cell-to-cell homotypic adhesion when its adhesion to the plastic of the culture flasks is prevented. In general, methods (Figure 2A) like the hanging drop culture technique, culturing on non-adherent surfaces[89,90,91,93,94] and more recently the magnetic levitation method - MLM, in which the cells are grown with nanoparticles and maintained in magnetic field culture[95], allow the formation of rounded cell aggregates (Figure 2B). The size of the spheroids varies depending on the number of cultured cells and the cell type. In addition, differences in the ability to establish cell-cell adhesions influence the formation of spheroids, which may be looser, with rough surface, or more firm (Figure 2B) [33,54,89,90]. The model allows the co-culturing of different cell types and it is interesting that in these spheroids the cells organize spontaneously, deposit extracellular matrix and form specific microenvironments[89,90,91,96,97]. Because of the diffusion from the periphery in contact with the culture medium to the center of the spheroid, a chemical gradient of O_2 and of cellular nutrients and metabolites is established along the radius of the spheroid (Figure 2C). Such diffusion can be improved with the use of bioreactors[53]. It should be said that various types of bioreactors are available today, but not all will be equally suitable.

The concentration of O_2 at the center of the spheroid correlates inversely with its size and, although variations are observed, spheroids of diameter above 500 µm generally develop apoptosis and necrosis of the cells located in the central region due to hypoxia. This central zone is surrounded by a region of quiescent cells and, more externally, there may be the formation of a proliferative zone, present in the spheroids of tumor cells, but virtually absent in those formed by non-transformed cells[54,89,90,91,93,94,97,98,99,100,101,102].

Central hypoxia and the various regions formed make the model especially advantageous in the area of oncology because it resembles nonvascularized tumor nodules[54,103]. On the other hand, the model may also favor the study of mechanisms of angiogenesis, when coculture with endothelial cells is established[101,104,105,106]. It is noteworthy that the integration of endothelial cells into the spheroid model of murine cardiomyocytes promoted cell survival, suggesting that there was improvement in the diffusion of the molecules present in the culture medium[105]. The model has been used with discernment and less frequently in the area of tissue bioengineering because of the possible development of central necrosis[106,107,108].

The similarity of the spheroids with avascularized tumor nodules led to their application in tests with chemotherapeutic drugs and new drugs. Several studies have shown that while monolayer tumor cells were sensitive to the action of several chemotherapeutic agents, when cultured in a spheroid model, they were resistant. On the other hand, some drugs proved to be effective only when the cells were in a 3D environment[54,103,109,110]. As a result of these differences, high-throughput screening assays for antitumor drug screening are becoming increasingly common[54,103,111,112,113,114,115]. At the same time, new analysis tools have been developed[116,117,118], which reinforces the potential of this model in the area of oncology.

Microfluidics and organ-on-a-chip culture models

The absence of blood vessels in most 3D models impacts the transport of fluids and small molecules[119,120]. In order to get more physiological 3D cell culture models, techniques that enable the creation of a spatial control of fluids, called microfluidic techniques, have been developed. Fluid flow control allows regulation of chemical gradients and, consequently, the development of specific microenvironments. Spatial control is the basis of the technique and the most sophisticated models involve multidisciplinary teams for the creation of micrometric standardized channels (micropatterning) in biopolymer molds[53,119,120,121].

Microfluidics, combined with 3D cell culture, enabled organ culture in chips, where microchannels filled with culture media interconnect specific-shaped wells that mimic the organs from which cells are withdrawn[122]. The cells are cultured in these specific wells and the interaction between the different cell types, which form the organs over the chip, takes place through the microchannels. This communication enables the systemic toxicological study[123], because the microfluidic technique allows the interconnection of chambers that mimic different organs, the so-called "body-on-a-chip"[124,125,126]. In addition to this on-a-chip system, mechanical forces may be applied to the substrate where the cells are cultured so as to generate stimuli mimicking those observed *in vivo*, like cardiac contraction and inhalation and exhalation pulmonary movements[127,128]. These mechanical stimuli modulate the cellular behavior making the system more physiological[129]. The potential of these models in the field of bioengineering and animal experimentation replacement is undeniable.

Advantages and limitations

There is no doubt that 3D cell culture models, by better mimicking the *in vivo* conditions, have promoted great progress in several areas of knowledge, including the influence of the microenvironment on various cellular properties (expression of genes and proteins, proliferation, death, migration and differentiation), morphogenesis, disease modeling, and cytotoxicity assays for the evaluation of new drugs[46,47,48,49,50,51,53,54,55,63,64,73]. In addition, such systems open the possibility of personalized studies, with the culturing of cells extracted from tissues *ex vivo* (primary cultures) in more physiological systems. Thus, every individual would have their own cells cultured in different models and their cellular response would be tested against drugs to be studied for the development of specific medicines or therapies for the individual in question[74,120,130,131,132,133].

The majority of these models allow easy manipulation, rapid hypothesis testing and real-time analysis when compared to *in vivo* models. Therefore, these systems are candidates for alternative methods to the use of experimental animals. In fact, in some areas, such as in cosmetic cytotoxicity, *in vitro* 3D models are being used in replacement of *in vivo* tests, banned in several countries. However, even in this area, some limitations of the model must be considered, as we shall see.

The choice of the model must take into account several factors, like the cell type and the application of the study, for example. This is fundamental for the reproducibility of the results. For instance, epithelial cells may form multiple layers or not, but they are founded anchor to the basement membrane and exhibit baso-apical polarization. On the other hand, some cell types are immersed in an extracellular matrix with particular characteristics. For example, osteoblasts are associated with a rigid matrix by association of hydroxyapatite crystals with collagen I molecules, in a process called matrix mineralization. That is, the type of model can have an impact on the results obtained. As we have seen, the components of the extracellular matrix include a wide variety of molecules that associate with each other and bind growth factors and cytokines, creating specific microenvironments. The differences in the composition of the matrix alter its three-dimensional organization, its mechanical properties and the chemical gradient[27,29,32]. Furthermore, the polymerization of some matrix compounds can create structural differences that impact cellular properties. For example, differences in fibril diameter and porosity of the collagen I matrix derived from rat tail or bovine dermis had an impact on the migration of tumor cell lineages[134]. Tatiana Coelho-Sampaio's group showed that pH changes the polymerization pattern of laminin, which, in turn, modifies the behavior of several cell types[68,135,136]. The variation in the polymerization of these biopolymers also occurs due to differences in their extraction, as in the case of matrigel, which is commercially available, but varies from batch to batch in its property of inducing tubulogenesis, which certainly has an impact on the reproducibility of the results[54,137]. That is, it is still a challenge to reproduce, under culture conditions, the mechanical properties, the porosity, the elasticity and the chemical gradient

of the extracellular matrix of tissues. Even in the case of engineered organs, the organ-on-a-chip models, mimicking the mass and volume ratio, or determining the various cell types that will be included are challenges that go beyond microfluidics and the engineering of a scaffold that mimic organs[138]. Thus, it is necessary to standardize the models, with well-defined criteria, to reduce variations in results. In addition, the cost must be taken into account, as well as the lack of controlled methods of large-scale evaluation of the effects obtained[53,54].

The use of 3D models of culture to replace the use of experimental animals should take into account that even the most complex models only partially represent the characteristics of organs and tissues. In other words, the microenvironment is simpler than that observed *in vivo* and therefore several pathophysiological mechanisms are not reproduced. *In vivo*, several systems interact and, none the less, the microenvironment is more complex, with contribution of cells of the nervous and lymphohematopoietic systems, besides the vascular system addressed above. Macrophages, dendritic cells, antigen-presenting cells, and lymphocytes, among others, create the microenvironment, but are absent in most models, compromising the evaluation of inflammatory effects and hypersensitivity[47,63]. Finally, the culture systems developed so far, in addition to spatial limitations, have temporal limitations, since they are systems that mimic short-lived events, whereas *in vivo* events succeed one another, in other words, they progress[47].

CONCLUSIONS

It is imperative to search for *in vitro* alternatives to the use of laboratory animals lined up with the policy of 3Rs that achieves, at the same time, reproducible and predictive results in clinical trials. In this sense, 3D cell culture systems, which mimic the complexity of tissues, have been developed. Overall, they enable cell-cell interactions and between these and the extracellular matrix, deposited by the cells themselves or derived from natural or synthetic matrix, which leads to a morphological organization of the cells and regulates their biological properties. Furthermore, physical and chemical gradients can form in these models, which also contributes to the modulation of cell behavior. The choice of the model must take into account not only the type of target tissue, but also the effect that it aims to ascertain, since the advantages, as well as the limitations, are typical of each model. The very innovative aspect of the 3D culture models is accompanied by challenges to its validation as a substitute model for the classical tests. A fundamental aspect, which is the reproducibility of the results, depends on the harmonization of protocols, the standardization of culturing methods in different laboratories, good practices in *in vitro* methods, the conduction of multi-laboratory tests, the automation of analysis methods and of adverse impact assessments, as recommended by international organizations, such as the Organization for Economic Co-operation and Development – OECD. Despite these challenges, 3D culture systems are a step toward models that are closer to tissue complexity. Therefore, they are also good candidates for alternative models to replace the use of experimental animals.

REFERENCES

1. U.S. Department of Enegy. Human genome project information archive 1999-2003. Washington, DC: U.S. Department of Enegy; 2015 [acesso 11 ago 2017]. Disponível em: http://web.ornl.gov/sci/techresources/Human_Genome/project/index.shtml
2. Venter JC, Smith HO, Adams MD. The sequence of the human genome. Clin Chem. 2015;61(9):1207-8. https://doi.org/10.1373/clinchem.2014.237016
3. Migliaccio AR, Quarto R, Piacibello W. Cell therapy: Filling the gap between basic science and clinical trials October 15-17, 2001, Rome, Italy. Stem Cells. 2001;21(3):348-56. https://doi.org/10.1634/stemcells.21-3-348
4. Nabel GJ. Genetic, cellular and immune approaches to disease therapy: past and future. Nat Med. 2004;10;135-41. https://doi.org/10.1038/nm990
5. Chien KR. Regenerative medicine and human models of human disease. Nature. 2008;453(7193):302-5. https://doi.org/10.1038/nature07037
6. Abdeen AA, Saha K. Manufacturing cell therapies using engineered biomaterials. Trends Biotechnol. 2017;35(10):971-82. https://doi.org/10.1016/j.tibtech.2017.06.008
7. Petricciani J, Hayakawa T, Stacey G, Trouvin JH, Knezevic I. Scientific considerations for the regulatory evaluation of cell therapy products. Biologicals. 2017;50:20-6. https://doi.org/10.1016/j.biologicals.2017.08.011
8. Twigger AJ, Scheel CH. Advances in stem cells and regenerative medicine: single-cell dynamics, new models and translational perspectives. Development. 2017;144(17):3007-3011. https://doi.org/10.1242/dev.153569
9. Gibson DG, Glass JI, Lartigue C. Creation of a bacterial cell controlled by a chemically synthesized genome. Science. 2010;329(5987):52-6. https://doi.org/10.1126/science.1190719
10. Annaluru N, Muller H, Mitchell LA et al. Total synthesis of a functional designer eukaryotic chromosome. Science. 2014;344(6179):55-8. https://doi.org/10.1126/science.1249252
11. Bosley KS, Botchan M, Bredernood AL, Carroll D, Charo RA, Corn J et al. CRISPR germline engineering—the community speaks. Nat Biotech. 2015;33(5):478-86. https://doi.org/10.1038/nbt.3227
12. Ma H, Marti-Gutierrez N, Park SW, Wu J, Lee Y, Suzuki K et al. Correction of a pathogenic gene mutation in human embryos. Nature. 2017;548(7668):413-19. https://doi.org/10.1038/nature23305
13. Edwards C. American biotechnology needs a strategic planning center. Nat. Biotech. 1983;1(1):7. https://doi.org/10.1038/nbt0383-7a
14. National Academies of Sciences, Engineering, and Medicine. Preparing for future products of biotechnology. Washington, DC: The National Academies; 2017.

15. Senado Federal (BR). Constituição da República Federativa do Brasil. Brasília, DF: Senado Federal; 1988.
16. Brasil. Lei nº 9.434, de 4 de fevereiro de 1997. Dispõe sobre a remoção de órgãos, tecidos e partes do corpo humano para fins de transplante e tratamento e dá outras providências. Diário Oficial União. 5 fev 1997.
17. Brasil. Lei nº 11.105, de 24 de março de 2005. Regulamenta os incisos II, IV e V do § 1o do art. 225 da Constituição Federal, estabelece normas de segurança e mecanismos de fiscalização de atividades que envolvam organismos geneticamente modificados - OGM e seus derivados, cria o Conselho Nacional de Biossegurança - CNBS, reestrutura a Comissão Técnica Nacional de Biossegurança - CTNBio, dispõe sobre a Política Nacional de Biossegurança - PNB, revoga a Lei nº 8.974, de 5 de janeiro de 1995, e a Medida Provisória nº 2.191-9, de 23 de agosto de 2001, e os arts. 5º, 6º, 7º, 8º, 9º, 10 e 16 da Lei nº 10.814, de 15 de dezembro de 2003, e dá outras providências. Diário Oficial União. 28 mar 2005.
18. Agência Nacional de Vigilância Sanitária - Anvisa. Resolução - RDC Nº 9, de 14 de março de 2011. Dispõe sobre o funcionamento dos Centros de Tecnolgia Celular para fins de pesquisa clínica e terapia e dá outras providências. Diário Oficial União. 16 mar 2011.
19. Basketter DA, Clewell H, Kimber I, Rossi A, Blaauboer B, Burrier R et al. A roadmap for the development of alternative (non-animal) methods for systemic toxicity testing - t4 report. ALTEX. 2012;29(1):3-91. https://doi.org/10.14573/altex.2012.1.003
20. Burden N, Sewell F, Chapman K. Testing chemical safety: what is needed to ensure the widespread application of non-animal approaches? PLoS Biol. 2015;13(5):e1002156. https://doi.org/10.1371/journal.pbio.1002156
21. Freshney RI. Culture of animal cells: a manual of basic technique. 5th ed. New York: John Wiley & Sons; 2005.
22. Meleady P, O'Connor R. General procedures for cell culture. In: Celis JE, editor. Cell biology: a laboratory handbook. 3rd ed. New York: Elsevier; 2006. p. 13-20.
23. Ebnet K, Suzuki A, Ohno S, Vestweber D. Junctional adhesion molecules (JAMs): more molecules with dual functions? J Cell Sci. 2004;117(Pt 1):19-29. https://doi.org/10.1242/jcs.00930
24. Wheelock MJ, Johnson KR. Cadherin-mediated cellular signaling. Curr Opin Cell Biol. 2003;15(5):509-14. https://doi.org/10.1016/S0955-0674(03)00101-7
25. Huveneers S, Rooij J. Mechanosensitive systems at the cadherin-F-actin interface. J Cell Sci. 2013;126(Pt 2):403-13. https://doi.org/10.1242/jcs.109447
26. Twiss F, Rooij J. Cadherin mechanotransduction in tissue remodeling. Cell Mol Life Sci. 2013;70(21):4101-16. https://doi.org/10.1007/s00018-013-1329-x
27. Hynes RO. The extracellular matrix: not just pretty fibrils. Science. 2009;326(5957):1216-9. https://doi.org/10.1126/science.1176009
28. Geiger B, Yamada KM. Molecular architecture and function of matrix adhesions. Cold Spring Harb Perspect Biol. 2011;3(5):a005033. https://doi.org/10.1101/cshperspect.a005033
29. Mouw JK, Ou G, Weaver VM. Extracellular matrix assembly: a multiscale deconstruction. Nat Rev Mol Cell Biol. 2014;15(12):771-85. https://doi.org/10.1038/nrm3902
30. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. Cell. 2006;126(4):677-89. https://doi.org/10.1016/j.cell.2006.06.044
31. Discher DE, Mooney DJ, Zandstra PW. Growth factors, matrices, and forces combine and control stem cells. Science. 2009;324(5935):1673-7. https://doi.org/10.1126/science.1171643
32. Humphrey JD, Dufresne ER, Schwartz MA. Mechanotransduction and extracellular matrix homeostasis. Nat Rev Mol Cell Biol. 2014;15(12):802-12. https://doi.org/10.1038/nrm3896
33. Rossi MI, Barros AP, Baptista LS, Garzoni LR, Meirelles MN, Takiya CM et al. Multicellular spheroids of bone marrow stromal cells: a three-dimensional in vitro culture system for the study of hematopoietic cell migration. Braz J Med Biol Res. 2005;38(10):1455-62. https://doi.org/10.1590/S0100-879X2005001000002
34. Wang N, Tytell JD, Ingber DE. Mechanotransduction at a distance: mechanically coupling the extracellular matrix with the nucleus. Nat Rev Mol Cell Biol. 2009;10(1):75-82. https://doi.org/10.1038/nrm2594
35. Clause KC, Barker TH. Extracellular matrix signaling in morphogenesis and repair. Curr Opin Biotechnol. 2013;24(5):830-3. https://doi.org/10.1016/j.copbio.2013.04.011
36. Bonfim DC, Dias RB, Fortuna-Costa A, Chicaybam L, Lopes DV, Dutra HS et al. PS1/γ-secretase-mediated cadherin cleavage induces β-catenin nuclear translocation and osteogenic differentiation of human bone marrow stromal cells. Stem Cells Int. 2016;2016:3865315. https://doi.org/10.1155/2016/3865315
37. Parmar K, Mauch P, Vergilio JA, Sackstein R, Down JD. Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. Proc Natl Acad Sci USA. 2007;104(13):5431-6. https://doi.org/10.1073/pnas.0701152104
38. Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci USA. 1995;92(12):5510-4. https://doi.org/10.1073/pnas.92.12.5510
39. Young EW, Beebe DJ. Fundamentals of microfluidic cell culture in controlled microenvironments. Chem Soc Rev. 2010 Mar;39(3):1036-48. https://doi.org/10.1039/b909900j
40. Bollenbach T, Heisenberg CP. Gradients are shaping up. Cell. 2015;161(3):431-2. https://doi.org/10.1016/j.cell.2015.04.009
41. Folkman J, Klagsbrun M, Sasse J, Wadzinski M, Ingber D, Vlodavsky I. A heparin-binding angiogenic protein—basic fibroblast growth factor—is stored within basement membrane. Am J Pathol. 1988;130(2):393-400.

42. Jones DL, Wagers AJ. No place like home: anatomy and function of the stem cell niche. Nat Rev Mol Cell Biol. 2008;9(1):11-21. https://doi.org/10.1038/nrm2319
43. Ferraro F, Celso CL, Scadden D. Adult stem cels and their niches. Adv Exp Med Biol. 2010;695:155-68. https://doi.org/10.1007/978-1-4419-7037-4_11
44. Voog J, Jones DL. Stem cells and the niche: a dynamic duo. Cell Stem Cell. 2010;6(2):103-15. https://doi.org/10.1016/j.stem.2010.01.011
45. Rossi MI, Borojevic R. Homeostasia dos tecidos. In: Gehn PM, organizador. Tratado de oncologia. São Paulo: Atheneu; 2013. Vol. 1, p. 232-44.
46. Griffith LG, Swartz MA. Capturing complex 3D tissue physiology in vitro. Nat Rev Mol Cell Biol. 2006;7(3):211-24. https://doi.org/10.1038/nrm1858
47. Yamada KM, Cukierman E. Modeling tissue morphogenesis and cancer in 3D. Cell. 2007;130(4):601-10. https://doi.org/10.1016/j.cell.2007.08.006
48. Baker BM, Chen CS. Deconstructing the third dimension: how 3D culture microenvironments alter cellular cues. J Cell Sci. 2012;125(Pt 13):3015-24. https://doi.org/10.1242/jcs.079509
49. Edmondson R, Broglie JJ, Adcock AF, Yang L. Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors. Assay Drug Dev Technol. 2014;12(4):207-18. https://doi.org/10.1089/adt.2014.573
50. Antoni D, Burckel H, Josset E, Noel G. Three-dimensional cell culture: a breakthrough in vivo. Int J Mol Sci. 2015;16(3):5517-27. https://doi.org/10.3390/ijms16035517
51. Ravi M, Paramesh V, Kaviya SR, Anuradha E, Solomon FD. 3D cell culture systems: advantages and applications. J Cell Physiol. 2015;230(1):16-26. https://doi.org/10.1002/jcp.24683
52. Oudin MJ, Weaver VM. Physical and chemical gradients in the tumor microenvironment regulate tumor cell invasion, migration, and metastasis. Cold Spring Harbor Lab Press. 2016;81:189-205. https://doi.org/10.1101/sqb.2016.81.030817
53. Duval K, Grover H, Han LH, Mou Y, Pegoraro AF, Fredberg J et al. Modeling physiological events in 2D vs. 3D cell culture. Physiology (Bethesda). 2017;32(4):266-77. https://doi.org/10.1152/physiol.00036.2016
54. Verjans ET, Doijen J, Luyten W, Landuyt B, Schoofs L. Three-dimensional cell culture models for anticancer drug screening: worth the effort? J Cell Physiol. 2018;233(4):2993-3003. https://doi.org/10.1002/jcp.26052
55. Birgersdotter A, Sandberg R, Ernberg I. Gene expression perturbation in vitro: a growing case for three-dimensional (3D) culture systems. Semin Cancer Biol. 2005;15(5):405-12. https://doi.org/10.1016/j.semcancer.2005.06.009
56. Olsavsky KM, Page JL, Johnson MC, Zarbl H, Strom SC, Omiecinski CJ. Gene expression profiling and differentiation assessment in primary human hepatocyte cultures, established hepatoma cell lines, and human liver tissues. Toxicol Appl Pharmacol. 2007;222(1):42-56. https://doi.org/10.1016/j.taap.2007.03.032
57. Weaver VM, Petersen OW, Wang F, Larabell CA, Briand P, Damsky C et al. Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. J Cell Biol. 1997;137(1):231-45. https://doi.org/10.1083/jcb.137.1.231
58. Wang F, Weaver VM, Petersen OW, Larabell CA, Dedhar S, Briand P et al. Reciprocal interactions between beta1-integrin and epidermal growth factor receptor in three-dimensional basement membrane breast cultures: a different perspective in epithelial biology. Proc Natl Acad Sci USA. 1998;95(25):14821-6. https://doi.org/10.1073/pnas.95.25.14821
59. Goodbye, flat biology? [Editorial]. Nature. 2003;424(6951):861. https://doi.org/10.1038/424861b
60. Abbott A. Cell culture: biology's new dimension. Nature. 2003;424(6951):870-2. https://doi.org/10.1038/424870a
61. Andrade LR, Arcanjo KD, Martins HS, dos Reis JS, Farina M, Borojevic R et al. Fine structure and molecular content of human chondrocytes encapsulated in alginate beads. Cell Biol Int. 2011;35(3):293-7. https://doi.org/10.1042/CBI20100273
62. Dhiman HK, Ray AR, Panda AK. Characterization and evaluation of chitosan matrix for in vitro growth of MCF-7 breast cancer cell lines. Biomaterials. 2004;25(21):5147-54. https://doi.org/10.1016/j.biomaterials.2003.12.025
63. Shamir ER, Ewald AJ. Three-dimensional organotypic culture: experimental models of mammalian biology and disease. Nat Rev Mol Cell Biol. 2014;15(10):647-64. https://doi.org/10.1038/nrm3873
64. Choi J, Lee EK, Choo J, Yuh J, Hong JW. Micro 3D cell culture systems for cellular behavior studies: culture matrices, devices, substrates, and in-situ sensing methods. Biotechnol J. 2015;10(11):1682-8. https://doi.org/10.1002/biot.201500092
65. Gospodarowicz D, Greenburg G, Birdwell CR. Determination of cellular shape by the extracellular matrix and its correlation with the control of cellular growth. Cancer Res. 1978;38(11 Pt 2):4155-71.
66. Madri JA, Williams SK. Capillary endothelial cell cultures: phenotypic modulation by matrix components. J Cell Biol. 1983;97(1):153-65. https://doi.org/10.1083/jcb.97.1.153
67. Kubota Y, Kleinman HK, Martin GR, Lawley TJ. Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. J Cell Biol. 1988;107(4):1589-98. https://doi.org/10.1083/jcb.107.4.1589
68. Freire E, Gomes FC, Linden R, Neto VM, Coelho-Sampaio T. Structure of laminin substrate modulates cellular signaling for neuritogenesis. J Cell Sci. 2002;115(Pt 24):4867-76. https://doi.org/10.1242/jcs.00173
69. Cukierman E, Pankov R, Stevens DR, Yamada KM. Taking cell-matrix adhesions to the third dimension. Science. 2001;294(5547):1708-12 https://doi.org/10.1126/science.1064829

70. Wolf K, Mazo I, Leung H, Engelke K, von Andrian UH, Deryugina EI et al. Compensation mechanism in tumor cell migration: mesenchymal-amoeboid transition after blocking of pericellular proteolysis. J Cell Biol. 2003;160(2):267-77. https://doi.org/10.1083/jcb.200209006
71. Simian M, Hirai Y, Navre M, Werb Z, Lochter A, Bissell MJ. The interplay of matrix metalloproteinases, morphogens and growth factors is necessary for branching of mammary epithelial cells. Development. 2001;128(16):3117-31.
72. Lee GY, Kenny PA, Lee EH, Bissell MJ. Three-dimensional culture models of normal and malignant breast epithelial cells. Nat Methods. 2007;4(4):359-65. https://doi.org/10.1038/nmeth1015
73. Lancaster MA, Knoblich JA. Organogenesis in a dish: modeling development and disease using organoid technologies. Science. 2014;345(6194):1247125. https://doi.org/10.1126/science.1247125
74. Bartfeld S, Clevers H. Stem cell-derived organoids and their application for medical research and patient treatment. J Mol Med (Berl). 2017;95(7):729-38. https://doi.org/10.1007/s00109-017-1531-7
75. Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature. 2009;459(7244):262-5. https://doi.org/10.1038/nature07935
76. Sato T, Clevers H. Growing self-organizing mini-guts from a single intestinal stem cell: mechanism and applications. Science. 2013;340(6137):1190-4. https://doi.org/10.1126/science.1234852
77. Lancaster MA, Renner M, Martin CA, Wenzel D, Bicknell LS, Hurles ME et al. Cerebral organoids model human brain development and microcephaly. Nature. 2013;501(7467):373-9. https://doi.org/10.1038/nature12517
78. Garcez PP, Loiola EC, Madeiro da Costa R, Higa LM, Trindade P, Delvecchio R et al. Zika virus impairs growth in human neurospheres and brain organoids. Science. 2016;352(6287):816-8. https://doi.org/10.1126/science.aaf6116
79. Xinaris C, Brizi V, Remuzzi G. Organoid models and applications in biomedical research. Nephron. 2015;130(3):191-9. https://doi.org/10.1159/000433566
80. Jenkinson EJ, Van Ewijk W, Owen JJ. Major histocompatibility complex antigen expression on the epithelium of the developing thymus in normal and nude mice. J Exp Med. 1981;153(2):280-92. https://doi.org/10.1084/jem.153.2.280
81. Thesleff I, Sahlberg C. Organ culture in the analysis of tissue interactions. Methods Mol Biol. 2008;461:23-30. https://doi.org/10.1007/978-1-60327-483-8_3
82. Hare KJ, Jenkinson EJ, Anderson G. In vitro models of T cell development. Semin Immunol. 1999;11(1):3-12. https://doi.org/10.1006/smim.1998.0151
83. Plum J, De Smedt M, Verhasselt B, Kerre T, Vanhecke D, Vandekerckhove B et al. Human T lymphopoiesis. In vitro and in vivo study models. Ann N Y Acad Sci. 2000;917(1):724-31. https://doi.org/10.1111/j.1749-6632.2000.tb05436.x
84. Green H, Kehinde O, Thomas J. Growth of cultured human epidermal cells into multiple epithelia suitable for grafting. Proc Natl Acad Sci USA. 1979;76(11):5665-8. https://doi.org/10.1073/pnas.76.11.5665
85. Yamaya M, Finkbeiner WE, Chun SY, Widdicombe JH. Differentiated structure and function of cultures from human tracheal epithelium. Am J Physiol. 1992;262(6 Pt 1):L713-24. https://doi.org/10.1152/ajplung.1992.262.6.L713
86. Karp PH, Moninger TO, Weber SP, Nesselhauf TS, Launspach JL, Zabner J et al. An in vitro model of differentiated human airway epithelia: methods for establishing primary cultures. Methods Mol Biol. 2002;188:115-37. https://doi.org/10.1385/1-59259-185-X:115
87. Pezzulo AA, Starner TD, Scheetz TE, Traver GL, Tilley AE, Harvey BG et al. The air-liquid interface and use of primary cell cultures are important to recapitulate the transcriptional profile of in vivo airway epithelia. Am J Physiol Lung Cell Mol Physiol. 2011;300(1):L25-31. https://doi.org/10.1152/ajplung.00256.2010
88. Azzopardi D, Haswell LE, Foss-Smith G, Hewitt K, Asquith N, Corke S et al. Evaluation of an air-liquid interface cell culture model for studies on the inflammatory and cytotoxic responses to tobacco smoke aerosols. Toxicol In Vitro. 2015;29(7):1720-8. https://doi.org/10.1016/j.tiv.2015.06.016
89. Mueller-Klieser W. Three-dimensional cell cultures: from molecular mechanisms to clinical applications. Am J Physiol. 1997;273(4 Pt 1):C1109-23. https://doi.org/10.1152/ajpcell.1997.273.4.C1109
90. Kunz-Schughart LA. Multicellular tumor spheroids: intermediates between monolayer culture and in vivo tumor. Cell Biol Int. 1999;23(3):157-61. https://doi.org/10.1006/cbir.1999.0384
91. Layer PG, Robitzki A, Rothermel A, Willbold E. Of layers and spheres: the reaggregate approach in tissue engineering. Trends Neurosci. 2002;25(3):131-4. https://doi.org/10.1016/S0166-2236(00)02036-1
92. Sutherland RM, McCredie JA, Inch WR. Growth of multicell spheroids in tissue culture as a model of nodular carcinomas. J Natl Cancer Inst. 1971;46(1):113-20. https://doi.org/10.1093/jnci/46.1.113
93. Kunz-Schughart LA, Kreutz M, Knuechel R. Multicellular spheroids: a three-dimensional in vitro culture system to study tumour biology. Int J Exp Pathol. 1998;79(1):1-23. https://doi.org/10.1046/j.1365-2613.1998.00051.x
94. Kelm JM, Fussenegger M. Microscale tissue engineering using gravity-enforced cell assembly. Trends Biotechnol. 2004;22(4):195-202. https://doi.org/10.1016/j.tibtech.2004.02.002

95. Haisler WL, Timm DM, Gage JA, Tseng H, Killian TC, Souza GR. Three-dimensional cell culturing by magnetic levitation. Nat Protoc. 2013;8(10):1940-9. https://doi.org/10.1038/nprot.2013.125
96. Garzoni LR, Adesse D, Soares MJ, Rossi MI, Borojevic R, de Meirelles MN. Fibrosis and hypertrophy induced by *Trypanosoma cruzi* in a three-dimensional cardiomyocyte-culture system. J Infect Dis. 2008 Mar;197(6):906-15. https://doi.org/10.1086/528373PMID:18279074
97. Barros AP, Takiya CM, Garzoni LR, Leal-Ferreira ML, Dutra HS, Chiarini LB et al. Osteoblasts and bone marrow mesenchymal stromal cells control hematopoietic stem cell migration and proliferation in 3D in vitro model. PLoS One. 2010;5(2):e9093. https://doi.org/10.1371/journal.pone.0009093
98. Acker H, Carlsson J, Mueller-Klieser W, Sutherland RM. Comparative pO2 measurements in cell spheroids cultured with different techniques. Br J Cancer. 1987;56(3):325-7. https://doi.org/10.1038/bjc.1987.197
99. Walenta S, Dötsch J, Bourrat-Flöck B, Mueller-Klieser W. Size-dependent oxygenation and energy status in multicellular tumor spheroids. Adv Exp Med Biol. 1990;277:889-93. https://doi.org/10.1007/978-1-4684-8181-5_102
100. Walenta S, Doetsch J, Mueller-Klieser W, Kunz-Schughart LA. Metabolic imaging in multicellular spheroids of oncogene-transfected fibroblasts. J Histochem Cytochem. 2000;48(4):509-22. https://doi.org/10.1177/002215540004800409
101. Timmins NE, Dietmair S, Nielsen LK. Hanging-drop multicellular spheroids as a model of tumour angiogenesis. Angiogenesis. 2004;7(2):97-103. https://doi.org/10.1007/s10456-004-8911-7
102. Schmitz A, Fischer SC, Mattheyer C, Pampaloni F, Stelzer EH. Multiscale image analysis reveals structural heterogeneity of the cell microenvironment in homotypic spheroids. Sci Rep. 2017;7:43693. https://doi.org/10.1038/srep43693
103. Friedrich J, Seidel C, Ebner R, Kunz-Schughart LA. Spheroid-based drug screen: considerations and practical approach. Nat Protoc. 2009;4(3):309-24. https://doi.org/10.1038/nprot.2008.226
104. Korff T, Augustin HG. Integration of endothelial cells in multicellular spheroids prevents apoptosis and induces differentiation. J Cell Biol. 1998;143(5):1341-52. https://doi.org/10.1083/jcb.143.5.1341
105. Garzoni LR, Rossi MI, Barros AP, Guarani V, Keramidas M, Balottin LB et al. Dissecting coronary angiogenesis: 3D co-culture of cardiomyocytes with endothelial or mesenchymal cells. Exp Cell Res. 2009;315(19):3406-18. https://doi.org/10.1016/j.yexcr.2009.09.016
106. Laschke MW, Menger MD. Spheroids as vascularization units: from angiogenesis research to tissue engineering applications. Biotechnol Adv. 2017;35(6):782-91. https://doi.org/10.1016/j.biotechadv.2017.07.002
107. Lee JH, Lee DH, Lee S, Kwon CH, Ryu JN, Noh JK et al. Functional evaluation of a bioartificial liver support system using immobilized hepatocyte spheroids in a porcine model of acute liver failure. Sci Rep. 2017;7(1):3804. https://doi.org/10.1038/s41598-017-03424-2PMID:28630420
108. Dinh PC, Cores J, Hensley MT, Vandergriff AC, Tang J, Allen TA et al. Derivation of therapeutic lung spheroid cells from minimally invasive transbronchial pulmonary biopsies. Respir Res. 2017;18(1):132. https://doi.org/10.1186/s12931-017-0611-0
109. Howes AL, Chiang GG, Lang ES, Ho CB, Powis G, Vuori K et al. The phosphatidylinositol 3-kinase inhibitor, PX-866, is a potent inhibitor of cancer cell motility and growth in three-dimensional cultures. Mol Cancer Ther. 2007;6(9):2505-14. https://doi.org/10.1158/1535-7163.MCT-06-0698
110. Barbone D, Yang TM, Morgan JR, Gaudino G, Broaddus VC. Mammalian target of rapamycin contributes to the acquired apoptotic resistance of human mesothelioma multicellular spheroids. J Biol Chem. 2008;283(19):13021-30. https://doi.org/10.1074/jbc.M709698200
111. Kunz-Schughart LA, Freyer JP, Hofstaedter F, Ebner R. The use of 3-D cultures for high-throughput screening: the multicellular spheroid model. J Biomol Screen. 2004;9(4):273-85. https://doi.org/10.1177/1087057104265040
112. Hirschhaeuser F, Menne H, Dittfeld C, West J, Mueller-Klieser W, Kunz-Schughart LA. Multicellular tumor spheroids: an underestimated tool is catching up again. J Biotechnol. 2010;148(1):3-15. https://doi.org/10.1016/j.jbiotec.2010.01.012
113. Tung YC, Hsiao AY, Allen SG, Torisawa YS, Ho M, Takayama S. High-throughput 3D spheroid culture and drug testing using a 384 hanging drop array. Analyst (Lond). 2011;136(3):473-8. https://doi.org/10.1039/C0AN00609B
114. Ivanov DP, Grabowska AM. Spheroid arrays for high-throughput single-cell analysis of spatial patterns and biomarker expression in 3D. Sci Rep. 2017;7:41160. https://doi.org/10.1038/srep41160
115. Ishiguro T, Ohata H, Sato A, Yamawaki K, Enomoto T, Okamoto K. Tumor-derived spheroids: relevance to cancer stem cells and clinical applications. Cancer Sci. 2017;108(3):283-9. https://doi.org/10.1111/cas.13155
116. Chen W, Wong C, Vosburgh E, Levine AJ, Foran DJ, Xu EY. High-throughput image analysis of tumor spheroids: a user-friendly software application to measure the size of spheroids automatically and accurately. J Vis Exp. 2014;(89):e51639. https://doi.org/10.3791/51639
117. Cisneros Castillo LR, Oancea AD, Stüllein C, Régnier-Vigouroux A. A novel computer-assisted approach to evaluate multicellular tumor spheroid invasion assay. Sci Rep. 2016;6(1):35099. https://doi.org/10.1038/srep35099
118. Zanoni M, Piccinini F, Arienti C, Zamagni A, Santi S, Polico R et al. 3D tumor spheroid models for *in vitro* therapeutic screening: a systematic approach to enhance the biological relevance of data obtained. Sci Rep. 2016;6(1):19103. https://doi.org/10.1038/srep19103
119. Lo RC. Application of microfluidics in chemical engineering. Chem Eng Process Tech. 2013;1:1002.

120. Duinen V, Trietsch SJ, Joore J, Vulto P, Hankemeier T. Microfluidic 3D cell culture: from tools to tissue models. Curr Opin Biotechnol. 2015;35:118-26. https://doi.org/10.1016/j.copbio.2015.05.002

121. Picollet-D'hahan N, Dolega ME, Liguori L, Marquette C, Le Gac S, Gidrol X et al. A 3D Toolbox to enhance physiological relevance of human tissue models. Trends Biotechnol. 2016;34(9):757-69. https://doi.org/10.1016/j.tibtech.2016.06.012

122. Chen Y, Chan HN, Michael SA, Shen Y, Chen Y, Tian Q et al. A microfluidic circulatory system integrated with capillary-assisted pressure sensors. Lab Chip. 2017;17(4):653-62. https://doi.org/10.1039/C6LC01427E

123. Bovard D, Iskandar A, Luettich K, Hong J, Peitsch MC. Organs-on-a-chip: A new paradigm for toxicological assessment and preclinical drug development. Toxicol Res Appl. 2017;1:1-16. https://doi.org/10.1177/2397847317726351

124. Sung JH, Esch MB, Prot JM, Long CJ, Smith A, Hickman JJ et al. Microfabricated mammalian organ systems and their integration into models of whole animals and humans. Lab Chip. 2013;13(7):1201-12. https://doi.org/10.1039/c3lc41017j

125. Maschmeyer I, Lorenz AK, Schimek K, Hasenberg T, Ramme AP, Hübner J et al. A four-organ-chip for interconnected long-term co-culture of human intestine, liver, skin and kidney equivalents. Lab Chip. 2015;15(12):2688-99. https://doi.org/10.1039/C5LC00392J

126. Miller PG, Shuler ML. Design and demonstration of a pumpless 14 compartment microphysiological system. Biotechnol Bioeng. 2016;113(10):2213-27. https://doi.org/10.1002/bit.25989

127. Shimizu T, Yamato M, Isoi Y, Akutsu T, Setomaru T, Abe K et al. Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces. Circ Res. 2002;90(3):e40. https://doi.org/10.1161/hh0302.105722

128. Huh D, Matthews BD, Mammoto A, Montoya-Zavala M, Hsin HY, Ingber DE. Reconstituting organ-level lung functions on a chip. Science. 2010;328(5986):1662-8. https://doi.org/10.1126/science.1188302

129. Bhise NS, Ribas J, Manoharan V, Zhang YS, Polini A, Massa S et al. Organ-on-a-chip platforms for studying drug delivery systems. J Control Release. 2014;190:82-93. https://doi.org/10.1016/j.jconrel.2014.05.004

130. Arrigoni C, Gilardi M, Bersini S, Candrian C, Moretti M. Bioprinting and Organ-on-chip applications towards personalized medicine for bone diseases. Stem Cell Rev. 2017;13(3):407-17. https://doi.org/10.1007/s12015-017-9741-5

131. Khazali AS, Clark AM, Wells A. A pathway to personalizing therapy for metastases using liver-on-a-chip platforms. Stem Cell Rev. 2017;13(3):364-80. https://doi.org/10.1007/s12015-017-9735-3

132. Jeppesen M, Hagel G, Glenthoj A, Vainer B, Ibsen P, Harling H et al. Short-term spheroid culture of primary colorectal cancer cells as an in vitro model for personalizing cancer medicine. PLoS One. 2017;12(9):e0183074. https://doi.org/10.1371/journal.pone.0183074

133. Fong EL, Toh TB, Yu H, Chow EK 3rd. Culture as a clinically relevant model for personalized medicine. SLAS Technol. 2017;22(3):245-53. https://doi.org/10.1177/2472630317697251

134. Wolf K, Te Lindert M, Krause M, Alexander S, Te Riet J, Willis AL et al. Physical limits of cell migration: control by ECM space and nuclear deformation and tuning by proteolysis and traction force. J Cell Biol. 2013;201(7):1069-84. https://doi.org/10.1083/jcb.201210152

135. Barroso MM, Freire E, Limaverde GS, Rocha GM, Batista EJ, Weissmüller G et al. Artificial laminin polymers assembled in acidic pH mimic basement membrane organization. J Biol Chem. 2008;283(17):11714-20. https://doi.org/10.1074/jbc.M709301200

136. Hochman-Mendez C, Cantini M, Moratal D, Salmeron-Sanchez M, Coelho-Sampaio T. A fractal nature for polymerized laminin. PLoS One. 2014;9(10):e109388. https://doi.org/10.1371/journal.pone.0109388

137. Auerbach R, Lewis R, Shinners B, Kubai L, Akhtar N. Angiogenesis assays: a critical overview. Clin Chem. 2003;49(1):32-40. https://doi.org/10.1373/49.1.32

138. Takebe T, Zhang B, Radisic M. Synergistic engineering: organoids meet organs-on-a-chip. Cell Stem Cell. 2017;21(3):297-300. https://doi.org/10.1016/j.stem.2017.08.016

Acknowledgements

The authors thank Dr. Anneliese Fortuna de Azevedo Freire da Costa, PhD, currently at the Institute of Traumatology-Orthopedics, Rio de Janeiro, RJ, Brazil and Dr. Araci Maria da Rocha Rondon, PhD, Institute of Medical Biochemistry, Federal University of Rio de Janeiro, Brazil, for the collaboration in the accomplishment of 3D cultures with human lineages of breast tumor. The authors also thank Prof. Tatiana Lobo Coelho-Sampaio for the information about the biological behavior of different cell lineages cultured on laminin matrix.

Conflict of Interest

Authors have no potential conflict of interest to declare, related to this study's political or financial peers and institutions.

Financing and conflict of interest

The work was funded by government agencies, Capes, Faperj and CNPq. The authors declare no conflict of interest.