Historical evolution of spheroids and organoids, and possibilities of use in life sciences and medicine

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Abstract

An impressive percentage of biomedical knowledge and advances were achieved through animal research and cell culture investigations. For drug testing and disease researches, both animal models and preclinical trials with cell cultures are extremely important, but they present some limitations, such as ethical concern and lack of representatively of human tissues and organs. Most cells are currently cultured using two-dimensional (2D) methods, but new and improved methods that implement three-dimensional (3D) cell culture techniques suggest convincing evidence that much more advanced experiments can be performed with more complex information. The environment and cell types in 3D culture can be manipulated to mimic tissue in vivo and provide more accurate data on cell-to-cell interactions; the cultivation techniques are based on a scaffold, which can be based on hydrogel or polymeric material, in addition there are techniques without using scaffold, such as suspended microplates, magnetic levitation and microplates for spheroids with ultra-low fixation coating. Even though 3D culturing is clearly incapable of replacing other current research types, they might continue to replace some unnecessary animal experimentation, as well as improve monolayer cultures. It is not even recommended or expected that 3D models substitute all other research types, but in regard to animal testing, they come in hand for the 3 Rs: Reduction, Refinement, Replacement. In this aspect, 3D culture emerges as valuable alternatives to the investigation of functional, biochemical and molecular aspects of human pathologies.

Introduction

For drug testing and disease researches, both animal models and preclinical trials with cell cultures are extremely important. An impressive percentage of biomedical knowledge and advances were only achieved through animal research and cell culture investigations. Nonetheless, experimenting with animals maintains many limits, as they often respond to pathogens in a different way than humans and also present a different course of the disease, in addition to the ethic concern when animal experiments are involved, besides the obvious ethic limitation when using laboratory lives (Morrisey and Hogan, 2010; Sakamoto, 2012). In regard to monolayer cell cultures, they succeed to imitate some human tissue, but fail to replicate human organs. Also, experiments using animals and cell cultures yield low success rates for new medicines; less than half of new drugs under testing fail due to lack of efficacy or concerns on safety before hitting the market (Arrowsmith and Miller, 2013; Langhans, 2018). Thus, it is understandable and necessary that researchers focus on investigating different research models that can replace the use of animals, yet allowing to simulate human structures as well as diseases more realistically. A rising number of researchers have started to switch from regular, bidimensional (2D) cell culturing to three-dimensional (3D) cultures; while 2D cultures are used for cell expansion and for some tissue-related investigations, the 3D arrangement allows a closer replication of the living situation (Montanez-Sauri et al., 2015). 3D cell culture also presents the possibility

of coculturing of different cell lineages in an extracellular matrix-like substance; similar to a full functional organ. In this aspect, they emerge as valuable alternatives to the investigation of functional, biochemical and molecular aspects of human pathologies.

Evolution of research with in vitro models

Cell culturing has enabled diverse advances in cell biology (Achilli et al., 2012), including what we know especially on extracellular signals (Bissell et al., 2002), and started being used during the XX century, by researches such as Harrison and Carrel, who aimed at better understanding cell behavior and function (Breslin and O'Driscoll, 2013). In 1907, Harrison successfully cultivated nervous cells isolated from amphibious spinal cord (Harrison, 1907); in 1912, Carrel proved it was possible to cultivate cells for longer periods since maintained under aseptic conditions and given enough nutrient supply (Carrel and Ingebrigsten, 1912). Some decades later, in 1951, George Otto Gey stablished the first human cultured cell line, derived from cervix cancer collected from a patient named Henrietta Lacks, the HeLa cells, a lineage that is employed to this day for cancer research (Ambrose, 2017; Earle et al., 1951; Ehrmann and Gey, 1956; Rahbari et al., 2009; Scherer et al., 1953). Monolayer cell culturing relies on the cell adherence into a flat surface, and on the constant addition of culture medium as nutritious resource to cells; the plaque is maintained at a temperature of 37°C and with 5% CO^2 flow, what resembles the human body conditions (Breslin and O'Driscoll, 2013).

In an in vivo situation, cells communicate to one another in addition to interacting with an extracellular matrix (ECM). They are in intimate contact to cells of the same type as well as other cell lineages, in a three-dimensional architecture, what constitutes the tissue or organ. Concerning drug testing, for example, the cell-cell connections in addition to cell-ECM interactions are mandatory in order to obtain a more valid replication of the in vivo context (Cushing and Anseth, 2007). Considering these aspects, although 2D cell cultures are still widely used for diverse researches, they present serious limitations. They can be improved using a transition to 3D culturing (Rimann and Graf-Hausner, 2012).

Tridimensional cultures are more sensitive to drugs due to the organization of surface receptors; also, cells are often in different stages, similar to in vivo conditions (Jensen and Teng, 2020). The petri dishes and the cellular monolayer are replaced by a gelatinous scaffold that resembles the ECM, and the culture, now unable to attach to the bottom of the plate, assembles in a resulting 3D morphology (Haisler et al., 2013). The resulting cultures allow co-cultivation of different cell lineages of the desired tissue or organ represent a more accurate replication of the in vivo condition, including morphology and functions, due to engineering and biology principles to construct the functional substitutes of the target-organ (Doryab et al., 2016). Because of the representation of histological and physiological key characteristics of the target-organ, 3D models can even be employed as research tools or even to replace damaged living tissue in human patients.

Despite the rapid boom observed in concern to organoid research, seen from the late 2000's on, methods using 3D cultivation dates back to the XX century. The cultivation of cell aggregations instead of the regular monolayer display was first mentioned in the 50s by George Gey, the same researcher who isolated HeLa cells. In a paper from 1956, Ehrmann and Gey report they cultivated different human cell lineages using collagen isolated from rat tail as a substrate, and originated cell agglomerates with no interference from the scaffold (Ehrmann and Gey, 1956). Two decades later, the first 3D culture was described by James Rheinwald and Howard Green, who developed in vitro tridimensional tissues derived from human progenitor cells (Rheinwatd and Green, 1975). In the late 80's and beginning of 90's, in vitro cultures derived from neuroblastomas (Hachitanda and Tsuneyoshi, 1994) and lung tissue (Zimmermann, 1987) were developed and referred to as organoids. Researchers from Rheinwald's and Green's lab continued working on 3D cell cultures and expanded their know-how. In the 90's, they successfully cultivated skin organoids from small amounts of primary cells of human donors, and the organoids were successfully used in the treatment of third-degree burn. Still in the 90's, the 3D cornea cultures were used to treat blindness in over 100 patients (Lindberg et al., 1993; Pellegrini et al., 1997). A brief description of the evolution of cell cultures, from the first cultivation to the generation of spheroids and organoids are in **Figure 1**.

A grand part of these 3D cultures is called "organoids" because the cells in a 3D environment spontaneously

organize themselves, forming complex histological structures, similar to those observed in the organs from which they are derived; for example, cells derived from mammary glands form structures similar to branched ducts or acini (Lee et al., 2007). The name "organoid" derives from the junction of the suffix "oid" (from Greek "eîdos", similar) to the word "organ", referring to a structure that resembles an organ; also, the name is also commonly taken as a small version of an organ, such as "mini-guts" (Sato et al., 2009) or "mini-tissues" (Almeqdadi et al., 2019).

Although there is a generalization of the use of the name "organoid" to refer to 3D cultures that presents multiple cell lineages in co-culture that are able to spatially organize and form clusters (Almeqdadi et al., 2019), there are some particularities among 3D models that must be taken into consideration. Both Sato and Clevers' laboratories referred to their models as intestinal organoids (Barker et al., 2007; Sato et al., 2009), but the cultures developed consisted purely of epithelial cell lines. Thus, in 2012, the Intestinal Stem Cell Consortium established some nomenclature guidelines: the term "organoid" refers to cultures containing multiple cell types, but must include epithelial- and mesenchymal-originated cells; epithelial-only 3D cultures must be referred to as "spheroid" (Almeqdadi et al., 2019; Mustata et al., 2013). The epithelial-mesenchymal interactions have been shows to guarantee the stability of the 3D culture at long-term (at least one year) (Almeqdadi et al., 2019) and is one of the characteristics presented by organoids, while not all spheroids are viable for longer periods in culture.

Spheroids and Organoids

The single-cell or multicellular spheroid model is based on the ability to homotypic cell-cell adhesion, when its adhesion to plastic of the culture flasks is prevented. In general, the methods to develop a spheroid include hanging drop technique, in which cells are cultivated suspended due to superficial tension; cultivation on non-adherent surfaces, with the use of a scaffold or gel; and the magnetic levitation method (MLM), in which cells are cultured with nanoparticles and kept in culture with a magnetic field, allowing the formation of cell clusters that are detached from dish bottom and manipulated with help of a magnet. The size of the spheres varies depending on the number of cells cultured 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 an irregular surface, or firmer. The model allows cultivation of single cell lineage as well as co-cultivation of different cell types, and presents the capacity of cells to self-organize spontaneously, deposit extracellular matrix and form specific microenvironments (Haisler et al., 2013; Kelm and Fussenegger, 2004; Layer et al., 2002; Mueller-Klieser, 1997). In oncology research, several trials have shown that while monolayer tumors were sensitive to the action of several chemotherapeutic agents, the same cells, when grown as spheroids, were resistant to them. On the other hand, some drugs were effective only when the cells were in a 3D environment. The central hypoxia and the diverse regions formed make the model especially advantageous in the oncology area because of its resemblance to non-vascularized tumor nodules. However, spheroids must be used with discernment due to the possible development of central necrosis (Laschke and Menger, 2017; Verjans et al., 2018).

Alternatively, organoids are artificial structures that represent fragments of functional organs created for in vitro studies, capable of exercising the primordial functions of the corresponding organ, and are more complex in comparison to spheroids. They must exhibit morphofunctional units of respective in vivo tissues (Simian and Bissell, 2017). Normally, organoids are composed by different cell types organized in a specific arrangement, and can be initiated from spheroids, as well as with help of barriers, with a layered deposition of different cell types. In the latter case, it is possible to control the culture composition, and as the cells continue to proliferate after the formation of the sphere, they present self-renewal and can survive at long-term, as previously stated. The spontaneous arrangement of cells to form the 3D structure increases the chances of presenting an organotypic phenotype, thus representing more realistically the in vivo tissue (Danielson et al., 2018). Furthermore, the possibility of using different cell types in the same organoid allows heterotypic intercellular contacts, providing additional advances in realistically representing tissue functionality and differentiation (Marin and Pagani, 2018; Simian and Bissell, 2017). A summary of characteristics from monolayer, spheroids and organoids are represented in **Figure 2**.

Getting started: Cell lineages for spheroid / organoid generation

Spheroids and organoids can be derived basically from two originating cell types: pluripotent stem cells (PSCs) or adult-tissue cells (ATCs). Concerning PSCs, they can be either embryonic stem cells or induced PSCs (iPSCs), de-differentiated from adult cells (mainly fibroblasts). Because of the greater differentiation capacity, the resulting 3D model presents diverse properties of the target-organ; they are highly expandable; and can survive transplantation (Hohwieler et al., 2017; Huang et al., 2015). On the other hand, 3D models derived from adult tissue can be either adult stem cells (ASCs) or fully differentiated cells, including immortalized cells and lineages commercially available. In either case, the generation depends on a range of growth factors (Boj et al., 2015), and are limited in regard to the possibility of originating developmental intermediates, but the starting cells are of easier access, such as small amounts of biopsy material.

A single ASC type can start an organoid, while usually a cell pool of PSCs is required (Ootani et al., 2009). As a consequence, ASCs-derived organoids develop with a spheroid phase, while organoids derived from PSCs are usually more complex models, able to represent complex organs such as brain parts (Monzel et al., 2016), kidney and lung (Lancaster et al., 2013; Pasca, 2018), and they also might undergo a spheroid stage prior to organoid completion. In the case of visceral models, the complete process from PSCs to organoids usually include a definitive endoderm induction (Clevers, 2016), through activin treatment usually; a subsequent anterior foregut induction; and then organ-specific progenitor spheroids (Dye et al., 2015).

The advantage of using PSCs to generate 3D cultures is that they can differentiate into almost every cell lineage present in the living organism, and associated with the correct differentiating and inducing factors, a variety of spheroids and organoids can be achieved. Also, the resulting culture are of great value in development and regenerative medicine (Qu et al., 2014; Weidgang et al., 2013; Xu et al., 2015), in addition to generating specific disease and patient conditions (Rezania et al., 2014; Sampaziotis et al., 2015). On the downside, the generation from PSCs can take an enormous amount of time because of the need of step-wise differentiations before obtaining the final 3D model. In addition, the model usually does not represent a mature tissue or organ.

For ATCs-derived models, the starting cells range from fetal cells (9+ weeks of development); stem cells isolated from bone marrow or adipose tissue, such as mesenchymal cells; adult progenitors isolated from the fully mature target-organ, whose cell fate is pre-determined; and fully differentiated cells. In regard to mesenchymal stem cells / stromal cells, which derive mesodermal-origin cells, they have been employed in clinical trials involving cell engineering (Aldahmash et al., 2012; Zhang et al., 2012), and because they secrete several important growth factors that support vascularization, immune response and extracellular matrix production, this results in a complex 3D culture. It has been previously reported that the microenvironment in the core of ASCs-derived spheroids can trigger more growth factor secretion, what in turn helps nurture the culture (Potapova et al., 2007). In this case of using differentiated cells, different cell types either isolated from the tissue (usually obtained by biopsy) or commercially acquired lineages are cultivated separately and later allocated in a co-cultivation system. The selected cell lines, for the generation of visceral models for instance, usually include epithelial and endothelial cells, and fibroblasts. The co-culture cells, once placed onto a scaffold such as Matrigel or collagen, tend to spontaneously agglomerate and grow, forming a 3D structure containing co-cultures.

Possibilities of use of 3D cultures

3D models such as spheroids and organoids present innumerous advantages as research tools, since they predict more vividly human responses due to the structural complexity, as well as mechanical, biochemical and integrative aspects (Bhatia and Ingber, 2014). Besides the capacity of offering microphysiological environments that mimic in vivo morphology and function, resembling tissues and organs, 3D models also allow researchers to monitor activities and responses in real time, such as medical interactions or external interferences. So far, considering the enormous range of tissues and organs that can be replicated using 3D models, both spheroids and organoids have proved to be handful for a diversity of researches, including development, drug screenings, disease mechanisms and organization aspects (Little, 2017).

Although the immediate assumption of use of 3D cultures in general for research, it must be considered that they also hold promise of being used as reparative tool in the case of damaged tissue (Perkhofer et al., 2018). Since it is possible to derive organoids from patients, and thus generate patient-specific cultures, and the fact that it is possible to genetically manipulate the culture, soon we hope to observe more cases in which 3D cultivated cells are used as regenerative medicine approaches, including genetic manipulation for diseases to which there is no effective treatment nor cure (Costa et al., 2018).

Nevertheless, some limiting aspects must be considered when working with 3D cultures. One of the main limitations is the ethic concern, such as the one that emerges in case of using PSCs of embryonic origin; under this aspect, it is possible to turn to iPSCs or ATCs (Simunovic and Brivanlou, 2017). Another point is the establishment of cerebral organoids with functional and firing neurons that present synapses, and how the cerebral-like culture could present moral status (Little, 2017; Munsie et al., 2017). The financial demand to generate and maintain some cultures must also be taken as a possible limitation (Verjans et al., 2018), since some of the required components, such as Matrigel and growth factors, along with some specific cell lineages, can end up in a large sum. In addition, some research groups report difficulties in regard to reproducibility and instability of culture, what depends directly on protocol patterning and validation, but also good laboratorial practices (Costa et al., 2018; Little, 2017; Perkhofer et al., 2018).

Final considerations and perspectives

Even though 3D culturing is clearly incapable of replacing other current research types, they might continue to replace some unnecessary animal experimentation, as well as improve monolayer cultures. It is not even recommended or expected that 3D models substitute all other research types, but in regard to animal testing, they come in hand for the 3 Rs: Reduction, Refinement, Replacement. The concept, postulated in 1959 by Russel and Burch, is still very pertinent, and proposes the need for alternative methods that present at least one of the following: Reduction in the total number of animals used; Refinement of experiments that endure pain or suffering; Replacement of experiments that can be performed without animals with the same representability (Costa et al., 2018; Kirk, 2018; Russell and Burch, 1959). Both spheroids and organoids represent, in this way, a further step in replicating organ characteristics in absence of animal suffering and with small ethic concerns, and promising candidates as realistic models that will thrive us to the medicine of the XXI century.

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Conflict of Interest

The authors declare no conflict of interest.

References

Figures and figure legends

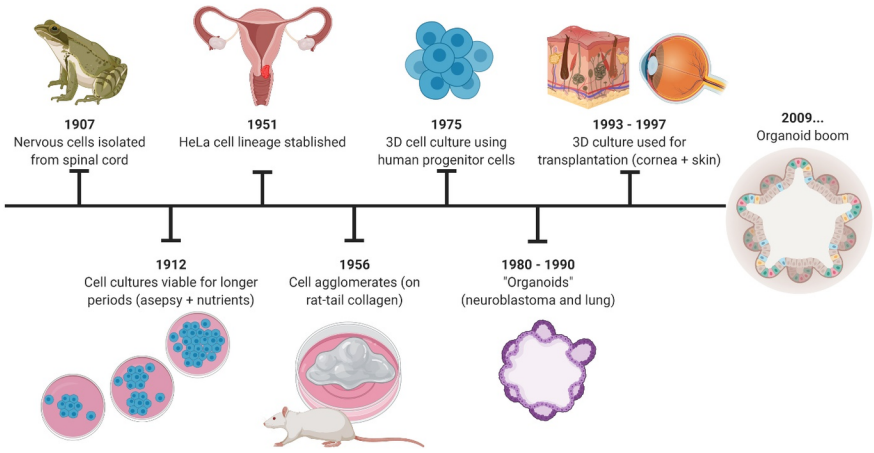

Figure 1: Evolution of in vitro cell culture, from the first successful cell isolation and cultivation in the early 1900's to the recent boom in organoid research, in the 2000's.

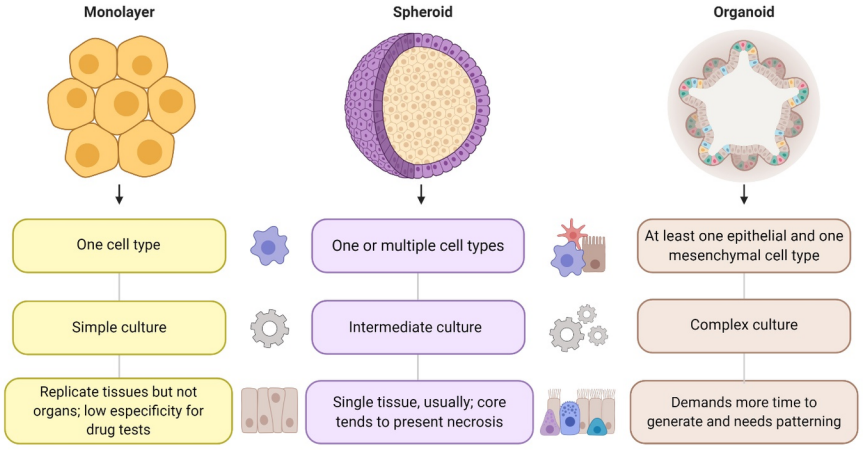

Figure 2: Main characteristics of Monolayer cell culture, Spheroids and Organoids, including number of cell types, complexity and functionality.