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Human Adipose-Derived Stem Cells Spheroids as Tools for Drug Discovery and Development

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ABSTRACT

Three-dimensional *in vitro* models are a new alternative to the classical approaches that can help to bridge the gap between traditional cell culture and live tissue. Besides, using human cells in a three-dimensional system has at least two important advantages comparing to animal models: (a) represents the same species; (b) offer the possibility for measurements of several cell physiology parameters. Adipose-derived stem cells are multipotent for mesodermical lineages, which mean that it is possible to build functional bone, cartilage, fat, and even to explore developmental toxicity, disease progression and drug discovery and development. As a mesenchymal stem cell source, adipose-derived stem cells are able to form cell aggregates also known as spheroids, with multidifferentiation capacity and superior tissue quality. The association between spheroid culture system and mesenchymal stem cell as cell source is a promising approach due to (a) the undifferentiated phenotype and the self-renewal capacity of stem cells, (b) better understanding

of the mechanisms ruling the differentiation fate, (c) improving more realistic engineering constructs to guide stem cells to a specific lineage, (d) more accuracy of outcomes predicting the target of drug discovery, (e) low costs. With the constantly improvement protocols for stem cell differentiation and hurdles regard ethical/medical harvesting somatic cells directly from the patient for its use in clinical testing, the application of adult mesenchymal stem cells make it more favorable for use in drug testing. Furthermore, obesity disorder could be modeled by making use of obese patient-derived adipose stem cells cultured as spheroids, as a system with remarkably potential to be similar to the disease development *in vivo*.

Keywords: Three-dimensional cell culture; Adipose-derived stem cells; Spheroids; Drug discovery and development; Obesity model

INTRODUCTION

Although the *in vitro* culture of mammalian cells in flat, rigid polystyrene dishes - where cells grow as monolayers in two-dimensions - contributed for advances in cell biology, physiology and cancer biology, tissues and organs are clearly organized in a complex three-dimensional system. Two-dimensional cell culture systems do not completely replicate all of the mechanical and biochemical aspects of the *in vivo* organization, which can jeopardize cell function [1]. One of the main differences between two-dimensional cell culture systems and the environment that cell experience *in vivo* is the interaction with an unusual substrate, and the lack of cell-cell and cell-extracellular matrix interactions, that could regulate normal cell function [2]. Because of this, many drugs that have positive effects on two-dimensional cultured cells do not show beneficial outcomes when tested *in vivo.* When considering the emerging area of tissue engineering, that aims to build tissues and organs from stem cells cultured *in vitro*, two-dimensional systems could not attend the demand to mimic tissue or organ function. Therefore, there is a clear need for *in vitro* models that faithfully replicate the tissue environment and three-dimensional culture systems. Three-dimensional *in vitro* models are a new alternative to the classical approaches that can help to bridge the gap between traditional cell culture and live tissue [1]. The prompt application of tissue engineering constructs is in fact the field of drug discovery and development.

CELL CULTURE SYSTEMS FOR DRUG DISCOVERY AND DEVELOPMENT

The release of the book “Principles of Human Experimental Technique” by William Russel and Rex Burch in 1959 have introduced the first action of the insurgent movement for experimental animals protection, representing a symbol in debate about use of animals for toxicity assays. From this point, 3R´s principle for use of animals is established (Reduction, Refinement and Replacement). In fact, alternative methods can be defined as such method used for reduce, refine or replace the use of animals in biomedical research, industrial assays or education. Animal-tested cosmetics were banned from European Community since 2009, and in 2013 even the imported cosmetics ingredients tested in animals were banned too [3,4]. Currently, the replacement of animal tests for *in vitro* or cell culture tests is mandatory for cosmetics area. The most common established *in vitro* tests contains a keratinocyte and an epithelial cell layer as a skin construct [5,6].

Besides the current ban of animal tests on cosmetic area, the ethical, cost, and time consuming of animal tests, make appropriate cell culture systems indispensable in drug discovery and development. Although tissues and organs are made of three-dimensional structure, the most applied methodology for drug discovery and development *in vitro* still remains on monolayer cell culture. Regardless of the important results generated by this method, cells grown in two-dimensional feature poorly mimetic a real life tissue. They main differ in morphology, cytoskeleton remodeling, cell core and flattening as the interactions between cell-cell and cell-matrix, which probably could lead to possible changes in gene expression profile and pursuant to protein synthesis [7].

The need for physiologic relevant cell culture systems is especially noted in drug screening. According to Tufts Center for the study of drug development [8] the cost to lead a drug to the market is approximately U1 billion and the cost to bring a compound to phase I on clinical trial could reach U$100 million. A potential drug in clinical trial phase I, has only 8% chances to be launched in the market [9]. The cost is even higher when there is need to withdraw the drug of the market. For example, the antibiotic trovafloxacin is one of a multiple examples of drugs that was removed for causing severe hepatotoxicity [10] costing U$8.5 billion in lawsuits for Pfizer [11].

Beyond the high costs and the gap of a more realist *in vitro* model for human conditions, animal tests remains the physiological system applied to assess toxicity and drug likeness. In an early study [12], it was found that combining the toxicity in rodents and non-rodents, through of testing 150 compounds from 12 large pharmaceutical companies, the predictive accuracy was only 50% of human hepatotoxicity. Furthermore, it is worth notice that probably several compounds that could have been safe in humans were discarded for its toxicity results in animals.

An important advantage of using three-dimensional cell culture is that the gene expression in this model is much closer to the clinical expression profile than when observed in two-dimensional models, enhancing the viability and maturity of platforms in three-dimensional systems for therapies test with greater clinical efficacy [13]. Besides, the IC50 for a class of drugs tested in three-dimensional cell culture is 100 times bigger than the IC50 for the same lineage of cells in monolayer cell culture (two-dimensions). Moreover, it is possible to obtain an increase of 7 to 25 times the economy of reagents compared to traditional two-dimensional cell culture systems on a large scale [14]. Therefore, three-dimensional cell culture becomes a very interesting model for drug development, improving the accuracy of the drug discovery once it is capable of reassembly the original tissue composition as engineering predictive *in vitro* models in addition to contribute to the reduction of animal testing and costs [15].

Finally, three-dimensional cell culture system using human cells has at least two important advantages comparing to animal models: (a) represents the same species; (b) offer the possibility for measurements of several cell physiology parameters. Although it is known that the mechanisms of toxicity often differs between species, animal testing remain considered gold standard [16].

Regarding human cell source, stem cells are the best choice for three-dimensional *in vitro* assays, since they have intrinsic capacity to differentiate into multiple cell types. Developmental toxicity, cytotoxicity and cell functional assays are three categories that stem cell toxicological screenings are classified [17]. Developmental toxicity evaluates if the tested drug has a possible negative effect on stem cell stemness. Immunopheno typing through flow cytometry to assess possible abnormalities on surface markers could be an example of screening to detect developmental toxicity [18]. Cytotoxicity assays explore viability levels after the compound exposure on the stem cells. Several studies have described the use of mesenchymal stem cells for evaluation of cytotoxicity after short and long time incubation with many types of nanoparticles such as silver, gold and titanium dioxide, as a linkage between cytotoxicity and the increased risk of developmental toxicity that exposure may cause in humans [19-21]. The functional assay is performed after stem cells differentiation *in vitro*, aiming to verify if the compound tested has any potential toxicological effects on these differentiated cells. The use of high throughput screenings could be an answerable test system [22].

Despite of stem cell-based toxicological assays be a major promise for drug testing, some questions still need to be resolved, such as standardization among the laboratories regarding stem cell differentiation protocols, a deeper study of stem cells responsiveness as well the exposure time of them to the compound that has been tested. Studies for ensuring the genotypic and phenotype stability of stem cells used in these tests over time such as validating the efficacy of pharmacokinetics and pharmacodynamics on human stem cells also need to be taken in concern [23].

Due this lack between early toxicity assays, ethical concerns and high costs, the three-dimensional cell culture system is an effective approach that bridges the gap between monolayer cell culture and animals testing, being an approach more reliable to predict *in vivo* human drug responsiveness [15], especially when using unique cells, such as cells isolated from patient biopsies. However, it is clear that to reduce, refine or replace (Principle of 3R's) is necessary to scientifically demonstrate the relevance and reliability of the alternative method. Despite of several advantages that three-dimensional systems using stem cells represents, there is still a need for characterization, improvement and standardize the protocols to maximize the use of this approach.

HUMAN ADULT STEM CELLS SOURCES FOR THREE-DIMENSIONAL CELL CULTURE SYSTEMS

In 2007, researchers showed that although the development process is considered irreversible, by introducing genes encoding specific transcription factors in already differentiated adult cells, they could return to pluripotent stage [24]. These are the induced pluripotent stem cells. The emergence of induced pluripotent stem cells brought new possibilities to deepen the understanding of stem cell biology and even reshape it, allowing the creation of disease models, understanding the basic biology with regard to the development and differentiation, identifying novel therapeutic targets and testing new therapies [25]. However, several obstacles have been identified since the beginning of nuclear reprogramming featuring induced pluripotent stem cells:

time consumption for maintenance of pluripotency, coming variability among different tissues and the most importantly, cells epigenetic memory [23].

Cells from different tissues show different susceptibilities to reprogramming, they may require more or less reprogramming factors for pluripotency. There are two main methods for obtaining induced pluripotent stem cells, which are the somatic cell nuclear transfer - an already differentiated enucleated cell receives an undifferentiated cell nucleus and passes to express its characteristics; and reprogramming by inserting transcription factors that induce an entire lineage to a specific destination [26]. There are evidences found by a comparison of the two main methods, that induced pluripotent stem cells have an epigenetic memory, characterized by a residual DNA methylation in low passage cultures which can limit the progress of differentiation [27].

Despite the new doors opened by the induced pluripotent stem cells to the understanding of cell biology and study of models of disease and drug screening, large controversies still surround their use. The major question is about how far the epigenetic memory, the genetic background and the characteristics that may arise during the reprogramming can influence cell fate [28] and consequently the results obtained in these studies. Given this uncertainties, adult stem cells, despite its more restricted differentiation spectrum capacity, provides a safer alternative and can reproduce more faithful the events that occurs *in vivo*.

In general, adult stem cells play an important role in maintaining homeostasis and integrity of adult tissues. They can be obtained from various tissues (in some are more abundant than others) and remain quiescent until the tissue suffers damage, being recruited to restore the integrity of the tissue. Among these, we highlight the mesenchymal stem cells that are a fraction of tissue stromal cells, multipotent for mesodermal lineages (osteocytes, adipocytes and chondrocytes) [29] besides having the ability to secrete factors that promote tissue remodeling, low immunogenicity and immunosuppressive [30].

Mesenchymal stem cells were first described in bone marrow [31]. However, they reside virtually in all adult tissues [32]. An important source of mesenchymal stem cells for use in tissue repair and regeneration are adipose-derived stem cells. This cell is a convenient choice for regenerative cell therapy due to easy access from patients with a minimally invasive method [33,34]. Adipose-derived stem cells are multipotent for mesodermical lineages, which means that it is possible to build functional bone, cartilage, fat, and even to explore developmental toxicity, disease progression and drug discovery and development.

As a mesenchymal stem cell source, adipose-derived stem cells are able to form cell aggregates with multidifferentiation capacity and superior tissue quality [35]. These stem cell aggregates also known as spheroids are formed mainly driven by adhesion molecules forces, as will be describe in more detail ahead. Moreover, spheroid-derived adipose stem cells secrete [36] and expressed higher levels of markers of angiogenic growth factors, such as Hepatocyte Growth Factor **(HGF)** and Vascular Endothelial Growth Factor **(VEGF)**, as well as pluripotency markers [37].

Considering mesodermal lineages, it was reported that the three-dimensional microenvironment found in spheroids displayed mature adipocyte with better morphologies as compared with monolayer cultures after adipogenic differentiation [38]. Besides, this system is also able to enhance chondrogenic differentiation due to reduction oxygen tension and mimicry of the chondrogenic niche spatial *in vivo* [39]. Adipose-derived stem cells spheroids are also able to increase differentiation potential and enhance matrix mineralization [40].

MESENCHYMAL STEM CELLS SPHEROIDS AS TOOLS FOR DRUG DISCOVERY AND DEVELOPMENT

In the past two decades, *in vitro* cell culture systems have progressed from monolayer (two-dimensional model) to three-dimensional systems. Three-dimensional systems include cells seeded into three-dimensional scaffolds, the classical approach of tissue engineering [41,42], cells dispersed into extracellular matrixes, for example hydrogels [43-45] and spheroids [46-48].

In the absence of a substrate to attach, mono-dispersed cells tends to aggregate and undergo a process called self-assembly, forming structures with a spherical shape, so-called spheroids [49]. Spheroids can be considered as a functional tissue, whereas cells recapitulate native tissue properties. Native tissue formation is driven by both assembling process and self-organization, which are fundamental mechanisms of *in vivo* development and generally occurs spontaneously. Cells in spheroids are immersed within a three-dimensional extracellular matrix network, produced and secreted by them, constituting a complex microenvironment that provides physical support, cell growth and the ability to construct tissue-like formations containing multiple cell layers [45].

In the regenerative medicine field, spheroids could also represent an innovative strategy to delivery cells in cell therapy protocols. In fact, aggregation of human mesenchymal stem cell into spheroids has demonstrated to be able to improve their therapeutic potential [50]. The ultimate goal in tissue engineering is replicate tissue-like properties to mimic the aspects of embryogenesis, morphogenesis, and organogenesis. For example, during embriogenesis, mesenchymal stem cells assemble into compact cellular aggregates, starting chondro-osteo differentiation. This condensation phenomenon is driven by mechanical forces and adhesion molecules [51]. Self-assembly of mesenchymal lineage *in vitro* resembles this initial stage.

The use of classic biodegradable solid scaffold-based approaches as a temporal template-like instructive support for cell attachment and morphogenesis represent a paradigm in tissue engineering [52,53]. However, this approach involves the mechanisms of biocompatibility and biodegradability [54]: *in vitro* cell seeding on a solid biocompatible and biodegradable scaffold with sequential, relatively slow, complete scaffold biodegradation and tissue morphogenesis leads to laborious, expensive, time consuming and commercially unsuccessful tissue engineering technology. This also limits process automation and large-scale industrial biofabrication of constructs, which are crucial for application for drug screening strategies, besides introducing

artificial components into the cell culture. Another limitation to the approach of using three-dimensional scaffolds is that they often represent a two-dimensional substrate, where the nature of cell interaction is two-dimensional [47]. Thus, the search for alternatives is becoming a technological imperative and spheroids could represent a scaffold-free promising strategy.

A variant of cell culture technologies for spheroids fabrication that can be scaled-up is based on micro-molded resections in nonadhesive hydrogels. Using this technology, up to 256 spheroids can be fabricated in a single well of a 12-wells plate, with a standardized shape and size [55]. Automation of this process is also possible and supports the development of a high-throughput drug screening platform based on spheroids.

Aggregation of mesenchymal stem cells into spheroids not only improves differentiation efficiency, but also enhances anti-inflammatory properties due to paracrine secretion of trophic factors [56]. Furthermore, this aggregation can be a procedure to increase their immune modulatory and pro-angiogenic properties as well as homing to injury sites [57].

The three-dimensional microenvironment of spheroids plays a critical role in translating *in vitro* culture mesenchymal stem cells to clinical applications due to ability to preserve their primitive stemnessproperties. Spheroids enhance mesenchymal stem cell biophysical and biochemical interaction that improve extrinsic and intrinsic cellular signals and has an influence in their biologic function [58]. This microenvironment enhances mesenchymal stem cell *in vitro* differentiation potential to adipogenic, chondrogenic and osteogenic lineages. The preservation of differentiation potentials in the three-dimensional cultures may be due to their natural cell-extracellular matrix interactions, which may play crucial regulatory roles in mesenchymal stem cell three-dimensional expansion [59].

At last, the association between spheroid culture system and mesenchymal stem cell as cell source is a promising approach due to (a) the undifferentiated phenotype and the self-renewal capacity of stem cells, (b) better understanding of the mechanisms ruling the differentiation fate, (c) improving more realistic engineering constructs to guide stem cells to a specific lineage, (d) more accuracy of outcomes predicting the target of drug discovery, (e) low costs. With the constantly improvement protocols for stem cell differentiation [17] and hurdles regard ethical/medical harvesting somatic cells directly from the patient for its use in clinical testing, the application of adult mesenchymal stem cells make it more favorable for use in drug testing.

ADIPOSE-DERIVED STEM CELL SPHEROIDS: IMPLICATION IN OBESITY COMPREHENSION AND ANTI-OBESITY DRUGS DISCOVERY.

Adipose tissue is a complex organ that regulates and coordinates energy homeostasis. Its disruption leads to the development of many diseases, including obesity, which is now considered a global epidemic. Besides, obesity is a major risk factor for other diseases as type II diabetes, hypertension and dyslipidemia [60]. Pharmaceutical and biotechnology industries have focused on the search of compounds that could treat obesity, but none of the compounds developed is efficient [61]. As a

result, there is a sense of urgency to the complex mechanisms that underlie obesity and potential treatments at the cellular level, as well as systems that could serve as a platform from anti-obesity drug testing.

At tissue level, obesity development is characterized by adipocyte hypertrophy, hyperplasia, an intense angiogenic activity and macrophage infiltration, leading to a low-chronic inflammation [62-67]. Planning strategies to treat and prevent obesity and other metabolic diseases associates to adipose tissue dysfunction requires the elucidation of the molecular pathways that regulates adipocyte development, metabolism and the cross-talk between adipocytes and other cell types of the scenario, like endothelial cells and macrophages. Employment of cellular models has provided many evidences. However, most *in vitro* models of adipose tissue function involve culturing cells as monolayers, which fail to mimic the three-dimensional complexity found *in vivo*, due to lack of complex cell-cell interaction and cellular signaling.

The study of adipocyte development involves a deeper understanding of adipogenesis. This begins with adipose stem cells, that entry the adipogenic differentiation pathway and generates pre-adipocytes, through a highly controlled molecular mechanism [68]. Indeed, adipocyte biology and development is being extensively studied through multiple cellular models and most of our knowledge about adipogenesis was obtained from studies of cell lines committed to the adipogenic lineage, including the preadipocytes 3T3-L1, derived from mouse embryos [69].

Three-dimensional culture systems were developed associating extracellular matrix and polymers with 3T3-L1 preadipocytes to study adipogenic differentiation in a more physiological context [70-72]. However, the use of scaffolds for adipose tissue *in vitro* engineering for drug testing could limit its application in a pharmaceutical industry compatible scale and add costs to the process. Thus, spheroids that mimic adipose tissue physiology could be fabricated in large scale and could also represent a less expensive technology than those based on scaffolds for microtissue engineering. Recently, a three-dimensional *in vitro* model of 3T3-L1 cultured on an elastin-like polypeptide-polyethyleneimine-coated surface for spheroid organization showed to be a promise for investigation of cellular responses to a pro-inflammatory stimulus [73].

However, a specific substance can show distinct effects on adipogenesis depending on cellular origin and context, maybe due to different physiological characteristics between species. While growth hormone promotes differentiation in murine preadipocyte cell lines, it inhibits differentiation of rat and human primary preadipocytes [74-76]. Therefore, the use of human cells could provide more reliable results when considering translating *in vitro* outcomes to the clinics. Cultivation of induced pluripotent stem cells could represent an alternative model for studying adipogenesis, but the disadvantages mentioned before in this chapter significantly limit its use. Human adult mesenchymal stem cells are multipotent for mesodermal lineages, which includes the differentiation capacity to the adipogenic lineage.

Among mesenchymal stem cell sources, human adipose-derived stem cells would be the best option when consider mimicking adipose tissue physiology *in vitro*, due to their intrinsic capacity to derive adipose tissue resident stromal cells like pre-adipocytes, adipocytes and endothelial cells [77]. Therefore, analysis of the differentiation process and mechanisms that regulate stem cell function could support the potential manipulation of these cells in regulating the obesity scenario. An important issue in differentiating stem cells is to recreate their native niche. The niche is the microenvironment composed of extracellular matrix components, soluble factors and neighboring cells [78], capable of regulating how stem cells participate in organogenesis, homeostasis and tissue repair [79]. Cell niche is three-dimensional in native tissues, so three-dimensional cell culture systems are crucial for adipose stem cell niche recapitulation *in vitro*.

The use of adult adipose-derived stem cells in three-dimensional spheroids culture models for recreating the adipose organ *in vitro* is mandatory, since they are the initiators of adipogenesis, influencing the adipocyte development, and may be key players in tissue homeostasis disruption in obesity [80]. It has been recently shown that obesity limits the differentiation capacity of adipose-derived stem cells and alters its secretory behavior, tending to a pro-inflammatory [81-84]. Ex-obese patients, who lost weigh after bariactric-surgery, also showed adipose-derived stem cells alterations, which seemed to have an adipogenic and inflammatory memory [81, 85]. Hence, obesity disorder could be modeled by making use of obese patient-derived adipose stem cells cultured as spheroids, as a system with remarkably potential to be similar to the disease development *in vivo*. In addition, spheroids formed by adipose stem cells from ex-obese patients could serve as a platform for (a) studying weigh regain mechanisms associated to adipose tissue that could serve as pharmacological targets and (b) for anti-weigh regain drugs development.

Furthermore, exposure to pollutants has being linked to obesity development and it was hypothesized that they could interact with internal biological mechanisms, modifying glucose and lipid metabolism, acting as endocrine disruptors [86-90]. It has been recently shown that they can modulate adipogenesis of human adipose stem cells cultured as monolayers [91]. Considering the above mentioned about differences in cellular behavior in two- and three-dimensional cell culture systems, assessment of toxicological effects of pollutants in adipose cells cultured in three-dimensional systems could represent a new area for obesity comprehension and treatment.

CONCLUDING REMARKS

Building tissues and organs *in vitro* from three-dimensional cell culture systems suitable for regeneration repair and damaged tissue substitution is the main goal of the expanding area of tissue engineering. Currently, three-dimensional spheroids cell culture technique is being considered a valuable tool for drug discovery and development because of its higher complexity than monolayers, focusing on physiology and pathology development *in vitro* studies (Figure 1). However, mature tissue phenotype in spheroids is mandatory for reliable results. Adipose tissue is a very attractive source for adult stem cells harvesting with applicability in manifold studies such developmental studies, nanotoxicological assays as on the bioengineering field. As a whole, three-dimensional spheroids formed from adipose cells represent a new arena that has

the potential for mimic the biology of adipose tissue and the mechanisms by which this tissue is involved in obesity development. Besides, the three-dimensional spheroid adipogenic platform could serve as a fundamental tool for *in vitro* assay development for the influence of endocrine disruptors in adipogenesis and for anti-obesity drug discovery.

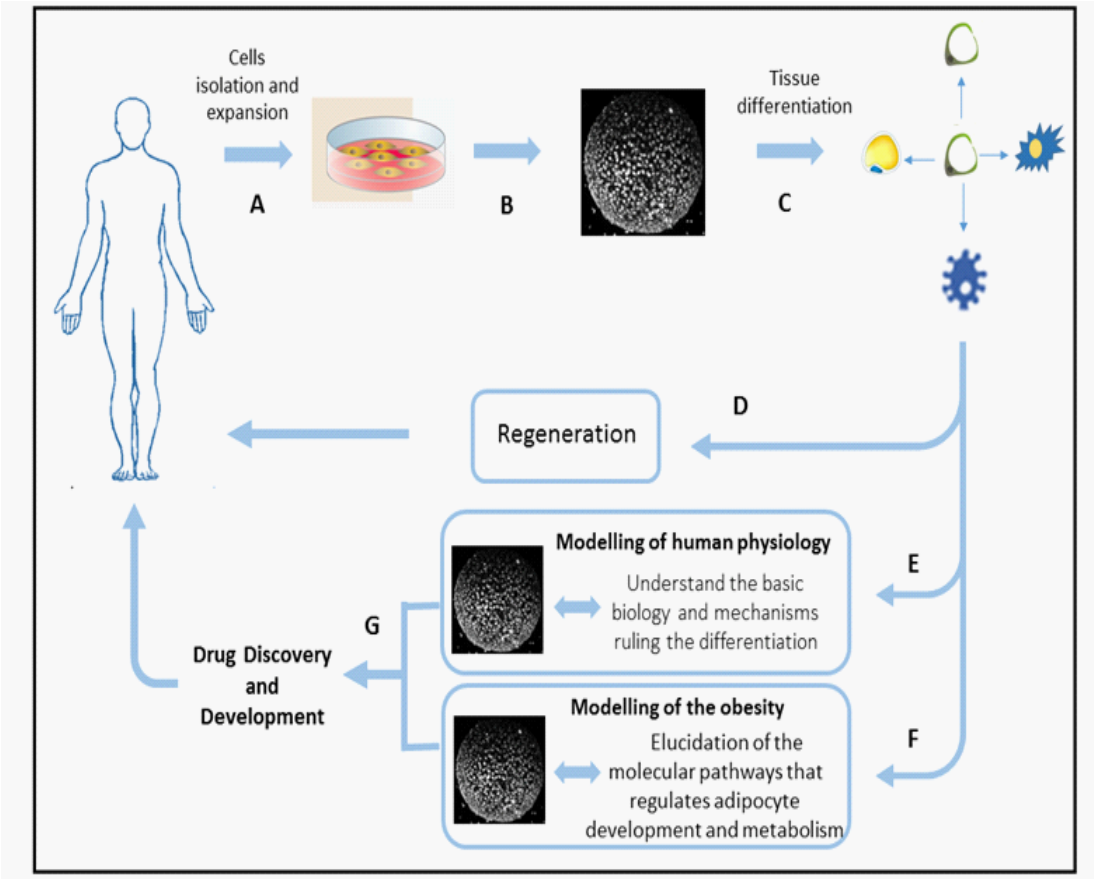

Figure 1: Main steps of tissue engineering for regeneration and drug discovery and development. One of the main contributions of three-dimensional systems is to promote the cell-cell and cell-extracellular matrix interactions, responsible for normal cell physiology. Besides, this tissue microenvironment is also able to improve chondrogenic, adipogenic and osteogenic differentiation, making them interesting for tissue engineering approaches that can support regeneration and drug discovery and development. The main steps of tissue engineering are: (A) Cells isolation from the patient and culture in monolayer for expansion (two-dimensional system); (B) Cell culture in spheroids (three-dimensional system); (C) Spheroids induction of differentiation in order to generate a mature tissue *in vitro* for: (D) Repair or even for replacement of a damaged tissue or organ, and tissue modeling to (E) Understand the basic biology of human physiology, as well as (F) Human disease physiology, like obesity; (G) Use of physio- and pathological models as a basis for drug discovery and development, which could attend patient drug treatment.2D: two-dimensions; 3D: three-dimensions.

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