Razavi Int J Med. 2014 May; (2): e17803. DOI: 10.30483/rijm.2014.118592
Published online 2014 May 31. **Review Article**

Three-Dimensional (3D) Cell Culture Conditions, Present and Future Improvements

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Received: January 24, 2014; **Revised:** February 26, 2014; **Accepted:** March 5, 2014

Context: Early development of many organs shows many morphological and molecular similarities (teeth, lung, hair, kidney and etc.). Fundamental questions in organogenesis are related to the identification of a simplified model which is able to mimic the molecular mechanisms involved in pattern organization and cell fate determination as well.
Evidence Acquisition: It is widely accepted that cells behave more natively if cultured in three-dimensional conditions. Advances in 3D non-destructive, non-invasive analysis methods and improvements in the multi-scale techniques and bioreactors to obtain test and culture 3D cell aggregates have been made. On the other hand, even if 3D aggregate culture methods are able to recapitulate in vitro the cell-extracellular matrix interactions properly observed in vivo, and the synthetic/natural matrix and scaffolds have biochemical and mechanical properties, in order to mimic the native extracellular matrix, both of these systems possesses some limitations and some methodological improvements are needed.
Results: The processes by which re-aggregated adult single cell types or adult and embryonic explanted tissues are able to recapitulate embryogenesis in vitro, when cultured in adhesion or embedded in 3D gels, is not surprising and is clearly under the control of a reminiscent cellular memory which recapitulates early developmental stages.
Conclusions: Our underlying hypothesis is that recapitulating the three-dimensional early embryonic structure, in order to reproduce better in vitro the three-dimensional morphogenetic-like re-arrangements, would improve cells differentiation, when in vivo transplanted; moreover, it could be used as a simplified cancer disease model and reliable drug evaluation method as well.

Keywords: Cell Culture Techniques; Organogenesis; Asymmetric Cell Division

1. Context

Embryonic stem cells (ESCs) are pluripotent and it is possible to differentiate them into almost every cell type of the human body. However, due to the ethical and legal issues, the use of ESCs is controversial (1), thus restricting their application for regenerative purposes in the clinic (2, 3). Unlike ESCs, adult stem cells (ASCs) (4, 5) and “induced” pluripotent stem (iPS) cells (6) have the potential to be used for the treatment of many different types of diseases, even if their stemness properties are different and potentially lower with respect to ESCs.

The extracellular matrix (ECM) interactions and cell-cell intercellular adhesion are strong inductors of cell fate decisions, providing all the morphogenetic signals to the stem cells. The ECM is a complex three-dimensional framework of macromolecules composed of glycosaminoglycans and fibrous proteins. This ECM structure provides mechanical support, adhesive interactions and is able to retain many growth factors. Moreover, ECM components, through integrin-mediated signaling events, are capable of directing cell differentiation; in addition, ECM proteolytic degradation is also able to release growth factors during matrix remodeling, influencing cell fate as well (7, 8).

Intracellular signaling cascades are the result of integrin ligation and growth factor binding to receptors that result in gene expression changes, modulating cell phenotype (8). Three-dimensional (3D) cellular models should ideally mimic the in vivo conditions in order to be helpful; however, cells are usually cultivated in vitro as monolayers and this culture condition does not permit to establish these functional requirements. To overcome this problem tissue engineering is being used as an alternative to animal models and 2D cell cultures with the intent to reproduce the structural and biochemical characteristics of 3D tissues.

Implication for health policy/practice/research/medical education:
To date research on three-dimensional cell systems is more vital and productive than ever, and such research effort mirrors the common need for improved and more refined models as link between in vitro 2D cell culture and organs in vivo. In this review we discuss the present achievements of the three-dimensional cell culture methods, a particular attention was devoted to these studies which are focused onto improve 3D aggregate formation methods, test culture and co-culture methods, also find and test suitable carrier structures and advance bioreactor technologies to obtain a sufficient nutrient supply.

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3D cell cultures have been helpful in biomedical research since the beginning of the past century. Using spherical re-aggregated cultures of embryonic cells was pioneered by Holtfreter (9) and Moscona (10, 11). During the past 5 years, there have been an exponential number of publications based on the 3D cell culture methods. The three methods: liquid suspension culture in bacterial-grade dishes, culture in methylcellulose semisolid media and culture in hanging drops, are usually performed to obtain in vitro 3D cell aggregates; these cell culture models, also known as embryoid bodies, spheroids, micromasses and mesen-spheres (8, 12), are used to study the cell differentiation and functionality of cell-cell adhesion in an organoid structure.

These models also include co-culture methods with different cell types, scaffolds and growth factors which have been developed with the intent to mimic the in vivo complex and dynamic interactions. In addition the ability to combine cells and matrices to generate 3D tissue engineered constructs, followed by their culture in bioreactors, has advanced rapidly in recent years becoming more advantageous for future clinical applications.

3D cultures may not replace the testing of biological mechanisms for their relevance to in vivo, as well as many scientific questions can be answered by simply using 2D cell culture methods. At the same time, as we will try to demonstrate in the present review, 3D in vitro approaches must be improved to permit a more true recapitulation of the in vivo organogenesis, in order to be used as election methods to evaluate and study stem cells differentiation mechanisms, as simplified cancer disease models, or a reliable drug evaluation method. In addition, these 3D structures could be the first step of a multistep approach, which involves both in vitro 3D culture and in vivo transplantation, to settle a future in tissue engineering.

2. Evidence Acquisition

2.1. Bi-Dimensional Vs Three-Dimensional Conditions

The intrinsic ability of stem cells to generate a diverse number of differentiated cells makes them a great resource for cell transplantation and tissue engineering applications. However, it is essential to find a way to efficiently control and direct their differentiation capability. Common formats to induce stem cells differentiation in vitro include monolayer culture on defined matrices (13) and co-culture with heterotypic cell types (14). In vivo and in vitro data clearly indicate the importance of physical, chemical and spatial cues from the local extracellular microenvironment in directing stem cell differentiation (15, 16).

Thus, current methods in tissue engineering have primarily focused on directing differentiation by adding soluble factors to the media in 2D conditions, or culturing in vitro spontaneous or induced cell aggregates on or within natural or synthetic extracellular matrix in 3D conditions. Both 2D and 3D culture models are highly robust and reproducible and offer the potential to study differentiation and cellular interactions. One major advantage of 3D cell cultures is their well-defined geometry, which mimics many of the hallmarks of early embryonic development; yet the 3D organization and structure of aggregates also presents unique challenges to direct effectively the differentiation of the cells and relate directly structure to function (17-19).

Moreover, it has been demonstrated in a recent study that mesenchymal stem cells (MSC) 3D aggregates formed using a forced aggregation technique and maintained in growth medium in suspension culture for extended time, exhibited no evidence of cell necrosis or differentiation, and retained their capacity for multi-lineage differentiation potential when dissociated. Thus, this study demonstrates that 3D culture system may circumvent limitations associated with the conventional monolayer cultures and maintain the differentiation potential of multipotent cells (19). In addition, in attempt to find a serum-free substitute to fetal calf serum containing media to induce stem cell proliferation, it has been demonstrated that human blood plasma based semi-solid medium has higher proliferation rates compared to a standard serum-free approach in 3D aggregate cultures (20). These reports thus confirm the importance of 3D culture methods over 2D methods either in scale up or differentiation conditions for the future clinical purposes.

2.2. Beyond Regenerative Medicine

2D cell culture systems and animal models are routinely used to assess toxicity, antitumoral activity for drug screening, and to gain insight into the diseases. While these systems are highly useful, there is growing recognition of the limitations with 2D cell culture and rodent models, because they are not able to correlate consistently to human clinical outcomes (21), leaving room for improved methodologies. As an example, animal models are widely used to study complex disease treatments including, but not limited to, treatments for viral (HIV), multibacillary (leprosy) and respiratory diseases, but they have proven to be an inaccurate model of clinical treatment outcomes (22, 23). This could be explained by the differences in the immunological response among humans and animals, or differences in metabolic and physiological responses. Conversely, 2D cell cultures, when used as model of drug screening give rise to concerns related to the absence of multi-dimensional inputs, the complex human tissue transport, and signaling systems.

Slow aggregation assays (SAA) are used to assess in vitro cell transformation and cell tumorigenicity, (24) exploiting the capacity of the transformed cells to form larger cell aggregates with respect to their untransformed counterpart, when cultured above an agar base (24, 25).

Three-dimensionally engineered biomimetic tissue models are extremely helpful due to their high fidelity in mimicking various native tissues, giving them a pivotal role during anti-cancer drug test, or the study of cancer cell development, interaction, and metastasis analysis (26, 27). Many studies indicate that gene expression profiles, cellular phenotypes, and differentiation capabilities are driven and directed by tissue architecture. In this view, drug evaluation process will gain tremendous benefits from the accurate predictions of cellular responses displayed by 3D engineered tissue models when exposed to anti-tumoral (28) and other drugs types (29) in vitro.

Nowadays 3D tissue models display reliable capabilities in predicting cellular responses to various therapeutic agents, and developing delivery methods with improved accuracy with respect to 2D counterparts (28, 29). While there is still room for refinement and improvement of this technology before it can be reliably applied to drug evaluation, today's 3D-engineered tissue models can act as in vitro living platforms for dynamic drug evaluation (27, 30) and simplified disease models (29).

2.3. 3D Analysis Methods

Standard approaches such as histology and quantitative polymerase chain reaction (qPCR), in situ hybridization and immunoblot techniques are commonly used to analyze 3D tissue engineered constructs (31-34), but are destructive and cannot be used with the intent to monitor tissue changes over time. This is mirrored in the increased number of samples needed to obtain statistically reliable results over the same number of time points.

Thus, the focus on creating clinically relevant engineered tissues needs new approaches for monitoring construct health during tissue development. This could be achieved having in mind that the technology should be in situ, non-invasive, and provide temporal and spatial information.

Recent and significant improvements in optical imaging methods, which are capable of probing endogenous cellular components in 3D specimens, have been used as a new approach to monitor tissue characteristics over time, substituting the need for destructive analysis (35-38). Examples of these noninvasive and non-destructive imaging methods are confocal microscopy, two-photon excitation fluorescence (TPEF), and fluorescence lifetime imaging (35, 39-41). Two-photon imaging has been used to analyze cellular differentiation grade and the metabolic activity in 3D engineered structures using different metabolic fluorophores as endogenous sources of contrast. Due to its characteristics this method is particularly suitable for drug and toxicology screening purposes that require repeated measurements of cell functions (34). Similarly, 3D tissue-engineered constructs transfected with a non-stable form of enhanced green fluorescent protein can be used to monitor exogenous stress.

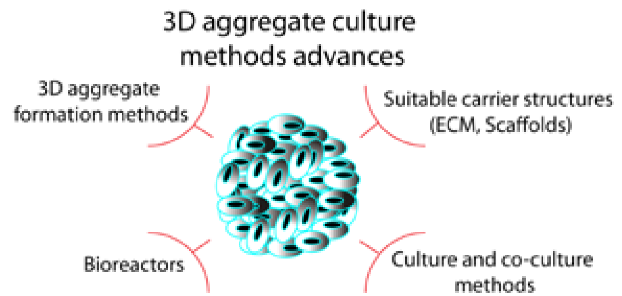

Figure 1. Summary of the 3D Aggregate Culture Methods Advances During Last Years

The performance and ability to predict the cellular response to stress in 3D tissue-engineered constructs of this method have been confirmed using compounds known to be pro-inflammatory or have pro-oxidative properties; however this method, with slight changes, could be a reliable method for detecting cellular differentiation in developing tissue engineered models (42). A new technique for in situ cell mapping, without the addition of exogenous probes exploits optical coherence microscopy (OCM) to quantify cell viability in engineered 3D tissues maintained during culture conditions. Cell viability was assessed by collecting and comparing time-lapse images of speckle generated by sub-cellular features and image cross-correlation was used to calculate the number of features that the final image has in common with the initial image, establishing the number of viable cells (43).

Tissue-engineered scaffolds are widely used in regenerative medicine. In this case, non-invasive and non-destructive imaging methods are needed to assess tissue-engineered constructs before and after seeding, and following in vivo implantation. It has also been reported that high-resolution magnetic resonance imaging (MRI) performed on stem cells is magnetically labeled by incubation with anionic citrate-coated iron-oxide nanoparticle, is a reliable technique to assess 3D structures and to validate cell-seeding procedures on scaffolds before and after in vivo implantation (44). Such new approaches, in combination with routinely performed molecular analysis, have the possibility to comprehend deeply 3D engineered constructs with regard to the cell shape and cellular environment, and determine gene expression and biological behavior of the cells as well.

2.4. 3D Culture Methods Advances

Tissue engineering is a biology driven approach by which bioartificial tissues are engineered through combining material science and biotechnology. Nowadays, research on 3D cell systems is more vital and productive than before and such research effort mirrors the com-

mon need for improved and more refined in vitro models as a link between in vitro 2D cell culture and organs in vivo. Many of these studies are focused on improved 3D aggregate formation methods (I), test culture and co-culture methods (II), also find and test suitable carrier structures (ECM matrix, scaffolds) (III) and advance bioreactor (IV) technologies to obtain a sufficient nutrient supply (Figure 1).

Advances in three dimensional culture methods have been made in order to improve 3D aggregate formation methods per se or by co-culturing different types of cells and by using suitable carrier structures (ECM, Scaffolds), as well as with the intention to improve their culture methods by using different bioreactors types.

2.4.1. 3D Aggregate Formation Methods

A novel method for constructing large numbers of deep concave wells in poly-dimethylsiloxane (PDMS) has been reported and applied for mass production of uniform sized 3D aggregates. 3D aggregates were successfully formed in the deep concave wells without loss of cells and laborious careful intervention required to refresh culture media. 3D-aggregates size was uniform, and their retrieving was done just by flipping over the chip. 3D aggregates formation and harvest were easy and safe to cells, showing high cell viability after completion of all processes (45, 46).

With the same intent, it was reported that in presence of pulsed ultrasound (pulsed acoustic field as well as with continuous ultrasound) particles and/or cells levitate in suspension and on aggregate. 3D aggregate rate can be modified in a controlled manner by carefully tuning the number of pulses and the repetition frequency (47, 48).

2.4.2. Culture and Co-Culture Methods

In the attempt to improve stem cell differentiation, it was also reported that the sequential application of 2D and 3D culture approach obtained significant improvement in cell differentiation and function (49). It is also known that the combination of 3D culture and co-culture with various cell types, namely 3D co-culture, can maintain the functions of primary cells as well as stem cell differentiation (50).

2.4.3. Find and Test Suitable Carrier Structures (ECM Matrix Scaffolds)

It is generally agreed that the cellular microenvironment plays an important role in modulating cell reorganization, migration, proliferation and differentiation. Among these microenvironment factors, there are chemical signaling factors, ECM composition and structure, physical and mechanical stimuli. Microenvironment factors that promote survival in the best way and define signaling pathways modulating the process of a specific tissue or organ differentiation have also been studied.

ECM consisting of structural and functional molecules represents a biological scaffolding material that provides structural support to cells and modulates cellular function and phenotype. Cell-ECM interactions are extremely important in a wide range of biological processes, from the formation of embryonic organs to pathological remodeling in disease states. It was demonstrated that 3D culture in presence of adherent extracellular matrix, or ECM single proteins favors cell differentiation. In one simple method, ECM substrate has been obtained by sequential chemical lysis and enzymatic digestion to isolate a thin, two-dimensional (2D), ECM substrate and used to improve stem cells differentiation cultured in 3D condition (51). Also it was demonstrated that 3D culture in the presence of culture media enriched with ECM single proteins supports cell differentiation. These studies also confirmed that ECM protein components of basement membrane reinforce the stability of epithelial structures during the epithelial-mesenchymal transition (EMT) in embryonic stem cells cultured as 3D aggregates (52, 53). In addition, it has been demonstrated that the addition of ECM proteins in culture medium favors differentiation and formation of a basement membrane-like in adult stem cells cultured in 3D aggregates conditions (33). These studies established that 3D culture conditions containing ECM components have an advantage in recapitulating tissue formation and interactions seen in embryonic development.

Conventional methods to test cell–ECM factors interactions involve depositing molecules of interest in cell culture dishes and then culturing cells on top or embedded within the 3D organized molecules. However, this approach is cumbersome and requires large quantities of reagents to screen many micro-environmental factors compositions. An alternative approach should involve micro-scale technologies, which can minimize the quantity of reagents used. In this regard, ECM protein microarrays have been demonstrated to be a micro-scale high-throughput approach for investigating and testing the effect of micro-environmental factors on stem cell differentiation (54, 55). In addition, aggregates confined to adhesive substrates and their subsequent retrieval from microwells for further experimentation and analysis could be beneficial for these ECM protein microarrays (54, 56).

Biologically-derived materials, such as alginate and agarose have been traditionally used for scaffolds construction but they may induce inflammation or fibrotic overgrowth which can impede nutrient and oxygen transport. According to Lee et al. 2013 (57) tissue-engineered scaffolds should (I) facilitate the delivery of tissue-specific cells to precise sites in the body, (II) maintain a 3D architecture that permits the formation of new tissues, (III) guide the development of new tissues with appropriate function and (IV) be reabsorbed by the cells (57).

Hydrogels are a highly attractive material used as syn-

thetic ECM substitutes due to their ability to mimic the hydrophilic nature of most tissues, investigate cell physiology, and fabricate tissues outside the organism. Intrinsically the polymeric hydrogel's structure possesses high water contents, transport of oxygen, nutrients and waste, as well as realistic transport of soluble factors. Furthermore, many hydrogels can be formed under mild, cytocompatible conditions and are easily modified to possess cell adhesion ligands, desired viscoelasticity and degradability (58). Besides their previously described properties, hydrogels with more porous structure that facilitate substrate colonization, oxygen and nutrients diffusion as well as incorporate sophisticated biochemical and mechanical cues, as mimics of the native extracellular matrix, are needed to provide a tissue specific induction.

In this attempt, D-mannitol crystals were mixed with photocrosslinkable methacrylamide chitosan (MAC) as a porogen to enhance pore size during the hydrogel formation (59). Alternatively, synthetic poly-ethylene glycol (PEG)-based hydrogels, even if they are not biodegraded, are easily functionalized, available at high purity, have controllable pore size and are extremely biocompatible. In addition, PEG hydrogels can be formed rapidly in a simple photo-crosslinking reaction that does not require application of non-physiological temperatures (60).

Additionally, it was also explored the possibility to use different materials to favor the contemporary regeneration of different tissues at the same time (61). Future developments of these methods and materials could be successfully employed to probe different matrix, growth factors and scaffold conditions in a large scale approach. This systematic approach will permit to better recapitulate embryonic signals, resulting in a 3D differentiation with improved tissues, ECM 3D organization and cell morphology, similarly to that happens in vivo.

2.4.4. Advanced Bioreactor Technologies to Obtain a Sufficient Nutrient Supply of the Cells

Cell-based tissue engineering have some intrinsic limitations due to the size of the cell-containing constructs that can be successfully cultured in vitro, highlighted by the low diffusion of nutritive molecules and oxygen into the interior of 3D aggregate culture. The typical arrangement of cells in aggregate culture conditions: proliferative cells at the periphery, intermediate zone with viable and clonogenic cells and a necrotic core in the center are clearly a limited 3D tissue model. This could be due to the hypoxia, lack of nutrients, accumulation of waste products, or low pH and is the "classical" disincentive for using 3D culture methods.

Bioreactors constitute and maintain physiological tissue conditions at a desired level, enhance mass transport rates and expose cultured cells to specific stimuli. Thus, bioreactor technologies providing appropriate biochemical and physiological regulatory signals guide cell differentiation and influence tissue specific function of 3D artificial tissues. Dynamic 3D perfusion culture in bioreactors has been demonstrated to be superior to induce maturation and prolong functions of primary adult cells and, at the same time, this approach has been tested on stem cells evidencing that it is prone to fully promote differentiation of stem cells and increase their vitality during differentiation process (62).

These studies have led to the increasing use of 3D aggregates in the present and their improvements and combinations will do better in the future.

2.5. Morphogenetic Rearrangements and 3D Culture

Early development of many organs shows many morphological and molecular similarities (teeth, lung, hair, kidney, etc.). Key questions in organogenesis involve the identification of a simplified model that recapitulates the molecular mechanisms of distinct pattern formation and cell fate determination. Simplifying, morphogenesis specifies which pathway and behaviour the cells have to follow in order to obtain a well ordered and functional shape, and this rearrangement arises because of changes in the cellular structure, morphology and cell polarity.

The complex morphogenetic rearrangements stem from a very limited number of cell biological processes: cell multiplication, cell expansion, elective cell death, cell migration, cell aggregation or condensation, cell fusion, mesenchymal-to-epithelial transition, keystone deformation and convergent extension (63, 64). In the course of executing the morphogenetic rearrangements, cells need to orient themselves in the plane of the whole tissue to which belongs.

In vivo, cells perceive signals from soluble growth factors, cell-cell interactions, components of the ECM, such as laminin and collagens, cell memory, and sense physical conditions, such as ECM stiffness, cell confinement and the orientation of such cell behaviors has a profound impact on the macroscopic rearrangements within the organism (Figure 2) (65).

Aggregate-assembly is a complex process involving multiple mechanisms working in concert; commonly during the first phase, the rounding-up of 3D aggregates into a spheroidal shape is a manifestation of liquid behavior (66). Subsequently, the process goes to completion by the effect of passive chemical forces resulting from binding of cadherins expressed on the cell surfaces (67), and by active chemical forces in which the cytoskeleton proteins play an important role in generation and stability of 3D aggregates (68-70).

3D aggregates cultured in solution are in a condition of equilibrium, in which they receive chemical signals from the medium but are not able to interchange physical signals due to the absence of physical contact with the environment. However, it has been reported that rotational and random movements are observed during morphogenesis in vitro and these random movements can occur in the absence of rotational external forces (71).

Previous evidence has been confirmed by another study which demonstrated that 3D aggregates cultured completely embedded in Matrigel matrix possesses such rotational capacity at least during early phases of mammary gland acini formation. Also in this case, authors observed no preference in rotation sense and few structures changed the direction of rotation during the course of morphogenesis (72). In addition, a recent study demonstrated that cells anchored in a 3D microenvironment showed significantly altered phenotypes, from altered cell adhesions, to cell migration and differentiation. Summarizing, in this condition cells were able to assemble an actin network in contact with a hard substrate, but remarkably, in presence of a softer substrate cytoskeleton, assembly was diminished also with respect to the cell cultured in 2D conditions (55). Cell confinement, and thus cell interaction with substrates, positive influence on cell spatial organization were also confirmed in a similar micro-patterned 3D cell culture method during the epithelial morphogenesis (54). These complex cell movements, interactions and morphogenetic-like rearrangements have been also demonstrated to permit to a single cell population of adult or embryonic stem cells to reorganize in ordered tissues once cultured in vitro in adhesion (73) as well as in organs by the recombination of the cells that constitute the organ itself (74).

The fact that these embryonic processes could be maintained in adult human cells in 3D gels is not surprising and organ/multi-tissue cultures have been commonly performed in semi-solid media since fifties (75).

For these reasons 3D cultures of stem cells completely embedded in natural matrix have become popular as 3D culture platforms both in differentiation approaches (76) as well as for proliferation aims (20); but even if these methods permit cell proliferation and favor cell differentiation due to the physical contact between matrix and 3D aggregate, these systems possess some limitations associated with perfusion of nutrients and oxygen. Morphogenesis and differentiation are influenced by a variety of factors comprising: soluble factor signaling, ECM interactions, cell–cell interactions, spatial confinement and orientation, matrix stiffness and cell memory.

3. Results

3.1. Morphogenesis and Cell Memory

The processes by which adult cells or adult and embryonic explanted tissues parts are able to recapitulate embryogenesis in adhesion or 3D gels are not well known, but are clearly under the control of a reminiscent cellular memory which recapitulates early developmental stages through genetic and epigenetic signaling.

In support to this observation, it has been demonstrated that early-passage induced Pluripotent Stem cells (iPs) preferentially differentiate into the cell lineage of the origin, negatively affecting their differentiation potential, as result of a temporary maintenance of their transcriptional and epigenetic memory (77). Morphogenesis of an organism results from coordination between cell interac tion, self-ordered properties, together with the action of internal and external physico-chemical stimuli.

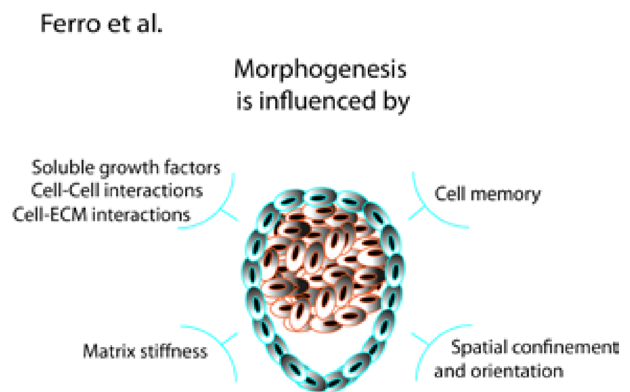

Figure 2. Factors Influencing in Vivo Morphogenesis and Differentiation

The genome's capacity to generate a form depend on many physico-chemical processes constituting a panoply of developmental mechanisms beyond the sheer capacity of the genome to co-ordinate the synthesis of specific RNA and protein molecules in time and space. Stem cells are capable of self-renewal and can give rise to the cells that have the potential to differentiate specialized cell types. The reminiscent transcriptional and epigenetic memory which recapitulates early developmental stages, found in differentiated single cell type or adult and embryonic (ASCs, ESCs) explanted tissues, is also present at variable degree in stem cells. This property depends on many factors, but needs to be recovered by adding correct external chemical and physical stimuli.

As already described, the rounding-up of 3D aggregates into a spheroidal shape is a manifestation of the liquid behavior, subsequently the process goes to completion by the effect of passive chemical forces resulting from binding of proteins expressed on cell surfaces and by active chemical forces in which cytoskeleton proteins play an important role in force generation and stability of 3D aggregates. Thus, when stem cells are cultured in differentiation conditions as 3D aggregates in suspension, the impossibility to perceive correctly the orientation within their behavior and the impossibility to physically interact with an external matrix, or belong to a whole tissue, do not allow to exploit these information to direct and support embryonic-like cellular rearrangements and presumably recall the stem cells reminiscent cellular memory. For these reasons, further improvements are needed to obtain 3D in vitro structures which are able to perform morphogenetic movements and rearrangements, perceive their spatial position, external chemical and physical stimuli acting as a “simplified-embryo”.

4. Conclusions

If the principal aim of regenerative medicine is regenerating in vitro, a transplantable organ with the shape and size of the physiological organ, previous studies suggest that the main limiting factor of 3D aggregate procedures is the size and shape of the 3D constructs. For these reasons, parallel studies have demonstrated that cell homing offers an alternative, especially with regard to the clinical translation, to methods of regeneration by cell transplantation. Cell homing is a well-recognized approach in tissue regeneration, the omission of cell isolation and ex vivo cell manipulation accelerates regulatory, commercialization, costs and clinical processes, (78-80) and this superiority is permitted by the fact that all cells, in growth-factor delivery or growth-factor-free scaffolds, are host derived differentiated endogenous cells.

On the other hand, even if this approach has given good results in terms of shape and size, it is far from the perfection, because the induced regeneration was partial and always lacked some tissues (61, 78-80). Failure may have been caused by the absence of a correct 3D framework between host cells, because even if cellular context was appropriate, it was unable to mimic the tissues relationship evidenced within organ's embryonic germ. On the contrary, 3D aggregate techniques have the capacity to reproduce in vitro, the cell-cell interactions occurring in vivo. Developing a 3D organization composed of phenotypically and morphologically different cells separated by basement membranes, could be useful if the aim of tissue engineering is regenerate in vitro, an organ germ which once in vivo transplanted will be able to recapitulate organogenesis and regenerate the entire organ. Despite advances in 3D non-destructive, non-invasive analysis methods and the improvements in the multi-scale techniques and bioreactors to obtain, test and culture 3D cell aggregates, some 3D methodological improvements are also needed.

Co-culturing 3D aggregates anchored to matrix scaffold or completely embedded in 3D matrix, could be helpful to maintain engineered aggregates in a static position, favor anchorage and morphogenetic-like movements, cell polarization as well as the cell layers adhesion, migration into the scaffold's matrix as well as layer specific differentiation. But the adhesion condition needed to colonize tailored scaffold's matrix, even if it favors cell migration, it will cause three-dimensionality loss; instead of the completely embedded approach, even if it permits physical contact between matrix and 3D aggregate, limits nutrients and oxygen perfusion.

In our opinion ongoing areas for improvement include proper 3D aggregate interaction with the external environment, aiming to recapitulate in vitro, some morphogenetic movements typically found in vivo. This condition could be helpful to mimic in vitro the in vivo tissue structure, permitting to exploit the physical, cell-cell, cell-ECM interactions and the gravity force to induce cellular morphogenetic-like movements, ameliorate ECM secretion and cell differentiation as well, dramatically improving current 3D tissue engineering methods, 3D cancer disease models and 3D drug screening methods as well.

Acknowledgements

We would like to thank Prof. Marcucio Ralph for his advice.

Authors' Contribution

Study concept and design: Ferro, Bahney, Spelat; acquisition of data: Ferro, Bahney, Spelat; drafting of the manuscript: Ferro, Bahney, Spelat; critical revision of the manuscript for important intellectual content: Ferro, Bahney, Spelat.

Financial Disclosure

Authors have no financial interests related to the material in the manuscript.

Funding/Support

This study has not received any grant support.

References

1. Denker HW. Potentiality of embryonic stem cells: an ethical problem even with alternative stem cell sources. *J Med Ethics.* 2006;**32**(11):665–71.
2. Shih CC, Forman SJ, Chu P, Slovak M. Human embryonic stem cells are prone to generate primitive, undifferentiated tumors in engrafted human fetal tissues in severe combined immunodeficient mice. *Stem Cells Dev.* 2007;**16**(6):893–902.
3. Prokhorova TA, Harkness LM, Frandsen U, Ditzel N, Schroder HD, Burns JS, et al. Teratoma formation by human embryonic stem cells is site dependent and enhanced by the presence of Matrigel. *Stem Cells Dev.* 2009;**18**(1):47–54.
4. Beltrami AP, Cesselli D, Beltrami CA. Pluripotency rush! Molecular cues for pluripotency, genetic reprogramming of adult stem cells, and widely multipotent adult cells. *Pharmacol Ther.* 2009;**124**(1):23–30.
5. Ferro F, Spelat R, D'Aurizio F, Puppato E, Pandolfi M, Beltrami AP, et al. Dental pulp stem cells differentiation reveals new insights in Oct4A dynamics. *PLoS One.* 2012;**7**(7).
6. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell.* 2006;**126**(4):663–76.
7. Czyz J, Wobus A. Embryonic stem cell differentiation: the role of extracellular factors. *Differentiation.* 2001;**68**(4-5):167–74.
8. Bratt-Leal AM, Carpenedo RL, McDevitt TC. Engineering the embryoid body microenvironment to direct embryonic stem cell differentiation. *Biotechnol Prog.* 2009;**25**(1):43–51.
9. Holtfreter J. A study of the mechanism of gastrulation. *J Exp Zool.* 1944;**95**(2):171–212.
10. Moscona A. Cell suspensions from organ rudiments of chick embryos. *Exp Cell Res.* 1952;**3**(3):535–9.
11. Moscona A. The Development in Vitro of Chimeric Aggregates of Dissociated Embryonic Chick and Mouse Cells. *Proc Natl Acad Sci U S A.* 1957;**43**(1):184–94.
12. Kurosawa H. Methods for inducing embryoid body formation: in vitro differentiation system of embryonic stem cells. *J Biosci Bioeng.* 2007;**103**(5):389–98.
13. Ying QL, Stavridis M, Griffiths D, Li M, Smith A. Conversion of embryonic stem cells into neuroectodermal precursors in adherent

monoculture. *Nat Biotechnol.* 2003;**21**(2):183–6.
14. Nakano T, Kodama H, Honjo T. Generation of lymphohematopoietic cells from embryonic stem cells in culture. *Science.* 1994;**265**(5175):1098–101.
15. Li X, Chen Y, Scheele S, Arman E, Haffner-Krausz R, Ekblom P, et al. Fibroblast growth factor signaling and basement membrane assembly are connected during epithelial morphogenesis of the embryoid body. *J Cell Biol.* 2001;**153**(4):811–22.
16. Komura H, Ogita H, Ikeda W, Mizoguchi A, Miyoshi J, Takai Y. Establishment of cell polarity by afadin during the formation of embryoid bodies. *Genes Cells.* 2008;**13**(1):79–90.
17. Daya S, Loughlin AJ, Macqueen HA. Culture and differentiation of preadipocytes in two-dimensional and three-dimensional in vitro systems. *Differentiation.* 2007;**75**(5):360–70.
18. Pineda ET, Nerem RM, Ahsan T. Differentiation patterns of embryonic stem cells in two- versus three-dimensional culture. *Cells Tissues Organs.* 2013;**197**(5):399–410.
19. Baraniak PR, McDevitt TC. Scaffold-free culture of mesenchymal stem cell spheroids in suspension preserves multilineage potential. *Cell Tissue Res.* 2012;**347**(3):701–11.
20. Greiner JF, Hauser S, Widera D, Muller J, Qunneis F, Zander C, et al. Efficient animal-serum free 3D cultivation method for adult human neural crest-derived stem cell therapeutics. *Eur Cell Mater.* 2011;**22**:403–19.
21. Kola I, Landis J. Can the pharmaceutical industry reduce attrition rates? *Nat Rev Drug Discov.* 2004;**3**(8):711–5.
22. Boucher RJ, Bromberg PA, Gatzy JT. Airway transepithelial electric potential in vivo: species and regional differences. *J Appl Physiol Respir Environ Exerc Physiol.* 1980;**48**(1):169–76.
23. Dunham SP. Lessons from the cat: development of vaccines against lentiviruses. *Vet Immunol Immunopathol.* 2006;**112**(1-2):67–77.
24. Rhim JS. Cell aggregation assay: a rapid means of evaluating and selecting in vitro transformed cells. *Cancer Detect Prev.* 1983;**6**(3):381–8.
25. Boterberg T, Bracke ME, Bruyneel EA, Mareel MM. Cell aggregation assays. *Methods Mol Med.* 2001;**58**:33–45.
26. Job CK. Nine-banded armadillo and leprosy research. *Indian J Pathol Microbiol.* 2003;**46**(4):541–50.
27. Achilli TM, Meyer J, Morgan JR. Advances in the formation, use and understanding of multi-cellular spheroids. *Expert Opin Biol Ther.* 2012;**12**(10):1347–60.
28. Sun P, Xu Y, Du X, Ning N, Sun H, Liang W, et al. An engineered three-dimensional gastric tumor culture model for evaluating the antitumor activity of immune cells in vitro. *Oncol Lett.* 2013;**5**(2):489–94.
29. Janorkar AV, Harris LM, Murphey BS, Sowell BL. Use of three-dimensional spheroids of hepatocyte-derived reporter cells to study the effects of intracellular fat accumulation and subsequent cytokine exposure. *Biotechnol Bioeng.* 2011;**108**(5):1171–80.
30. Peck Y, Wang DA. Three-dimensionally engineered biomimetic tissue models for in vitro drug evaluation: delivery, efficacy and toxicity. *Expert Opin Drug Deliv.* 2013;**10**(3):369–83.
31. Ferro F, Spelat R, D'Aurizio F, Falini G, De Pol I, Pandolfi M, et al. Acellular bone colonization and aggregate culture conditions diversely influence murine periosteum mesenchymal stem cell differentiation potential in long-term in vitro osteoinductive conditions. *Tissue Eng Part A.* 2012;**18**(13-14):1509–19.
32. Ferro F, Falini G, Spelat R, D'Aurizio F, Puppato E, Pandolfi M, et al. Biochemical and biophysical analyses of tissue-engineered bone obtained from three-dimensional culture of a subset of bone marrow mesenchymal stem cells. *Tissue Eng Part A.* 2010;**16**(12):3657–67.
33. Ferro F, Spelat R, Falini G, Gallelli A, D'Aurizio F, Puppato E, et al. Adipose tissue-derived stem cell in vitro differentiation in a three-dimensional dental bud structure. *Am J Pathol.* 2011;**178**(5):2299–310.
34. Ward A, Quinn KP, Bellas E, Georgakoudi I, Kaplan DL. Noninvasive metabolic imaging of engineered 3D human adipose tissue in a perfusion bioreactor. *PLoS One.* 2013;**8**(2).
35. Rice WL, Kaplan DL, Georgakoudi I. Quantitative biomarkers of stem cell differentiation based on intrinsic two-photon excited fluorescence. *J Biomed Opt.* 2007;**12**(6):60504.
36. Georgakoudi I, Rice WL, Hronik-Tupaj M, Kaplan DL. Optical spectroscopy and imaging for the noninvasive evaluation of engineered tissues. *Tissue Eng Part B Rev.* 2008;**14**(4):321–40.
37. Quinn KP, Bellas E, Fourligas N, Lee K, Kaplan DL, Georgakoudi I. Characterization of metabolic changes associated with the functional development of 3D engineered tissues by non-invasive, dynamic measurement of individual cell redox ratios. *Biomaterials.* 2012;**33**(21):5341–8.
38. Rice WL, Kaplan DL, Georgakoudi I. Two-photon microscopy for non-invasive, quantitative monitoring of stem cell differentiation. *PLoS One.* 2010;**5**(4).
39. Dittmar R, Potier E, van Zandvoort M, Ito K. Assessment of cell viability in three-dimensional scaffolds using cellular auto-fluorescence. *Tissue Eng Part C Methods.* 2012;**18**(3):198–204.
40. Konig K, Riemann I. High-resolution multiphoton tomography of human skin with subcellular spatial resolution and picosecond time resolution. *J Biomed Opt.* 2003;**8**(3):432–9.
41. Provenzano PP, Rueden CT, Trier SM, Yan L, Ponik SM, Inman DR, et al. Nonlinear optical imaging and spectral-lifetime computational analysis of endogenous and exogenous fluorophores in breast cancer. *J Biomed Opt.* 2008;**13**(3):31220.
42. Canton I, Sarwar U, Kemp EH, Ryan AJ, MacNeil S, Haycock JW. Real-time detection of stress in 3D tissue-engineered constructs using NF-kappaB activation in transiently transfected human dermal fibroblast cells. *Tissue Eng.* 2007;**13**(5):1013–24.
43. Dunkers JP, Lee YJ, Chatterjee K. Single cell viability measurements in 3D scaffolds using in situ label free imaging by optical coherence microscopy. *Biomaterials.* 2012;**33**(7):2119–26.
44. Poirier-Quinot M, Frasca G, Wilhelm C, Luciani N, Ginefri JC, Darrasse L, et al. High-resolution 1.5-Tesla magnetic resonance imaging for tissue-engineered constructs: a noninvasive tool to assess three-dimensional scaffold architecture and cell seeding. *Tissue Eng Part C Methods.* 2010;**16**(2):185–200.
45. Jeong GS, Jun Y, Song JH, Shin SH, Lee SH. Meniscus induced self organization of multiple deep concave wells in a microchannel for embryoid bodies generation. *Lab Chip.* 2012;**12**(1):159–66.
46. Kang E, Choi YY, Jun Y, Chung BG, Lee SH. Development of a multilayer microfluidic array chip to culture and replate uniform-sized embryoid bodies without manual cell retrieval. *Lab Chip.* 2010;**10**(20):2651–4.
47. Bazou D, Castro A, Hoyos M. Controlled cell aggregation in a pulsed acoustic field. *Ultrasonics.* 2012;**52**(7):842–50.
48. Vanherberghen B, Manneberg O, Christakou A, Frisk T, Ohlin M, Hertz HM, et al. Ultrasound-controlled cell aggregation in a multi-well chip. *Lab Chip.* 2010;**10**(20):2727–32.
49. Ramasamy TS, Yu JS, Selden C, Hodgson H, Cui W. Application of three-dimensional culture conditions to human embryonic stem cell-derived definitive endoderm cells enhances hepatocyte differentiation and functionality. *Tissue Eng Part A.* 2013;**19**(3-4):360–7.
50. Nagamoto Y, Tashiro K, Takayama K, Ohashi K, Kawabata K, Sakurai F, et al. The promotion of hepatic maturation of human pluripotent stem cells in 3D co-culture using type I collagen and Swiss 3T3 cell sheets. *Biomaterials.* 2012;**33**(18):4526–34.
51. Zwolinski CM, Ellison KS, Depaola N, Thompson DM. Generation of cell-derived three dimensional extracellular matrix substrates from two dimensional endothelial cell cultures. *Tissue Eng Part C Methods.* 2011;**17**(5):589–95.
52. Fujiwara H, Hayashi Y, Sanzen N, Kobayashi R, Weber CN, Emoto T, et al. Regulation of mesodermal differentiation of mouse embryonic stem cells by basement membranes. *J Biol Chem.* 2007;**282**(40):29701–11.
53. Shi Y, Kirwan P, Smith J, Robinson HP, Livesey FJ. Human cerebral cortex development from pluripotent stem cells to functional excitatory synapses. *Nat Neurosci.* 2012;**15**(3):477–86.
54. Rodriguez-Fraticelli AE, Auzan M, Alonso MA, Bornens M, Martin-Belmonte F. Cell confinement controls centrosome positioning and lumen initiation during epithelial morphogenesis. *J Cell Biol.* 2012;**198**(6):1011–23.
55. Ochsner M, Textor M, Vogel V, Smith ML. Dimensionality controls cytoskeleton assembly and metabolism of fibroblast cells

in response to rigidity and shape. *PLoS One*. 2010;**5**(3).
56. Tekin H, Anaya M, Brigham MD, Nauman C, Langer R, Khademhosseini A. Stimuli-responsive microwells for formation and retrieval of cell aggregates. *Lab Chip*. 2010;**10**(18):2411–8.
57. Lee SJ, Atala A. Scaffold technologies for controlling cell behavior in tissue engineering. *Biomed Mater.* 2013;**8**(1):10201.
58. Tibbitt MW, Anseth KS. Hydrogels as extracellular matrix mimics for 3D cell culture. *Biotechnol Bioeng.* 2009;**103**(4):655–63.
59. Lutolf MP, Hubbell JA. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. *Nat Biotechnol.* 2005;**23**(1):47–55.
60. Bernard AB, Lin CC, Anseth KS. A microwell cell culture platform for the aggregation of pancreatic beta-cells. *Tissue Eng Part C Methods*. 2012;**18**(8):583–92.
61. Abukawa H, Zhang W, Young CS, Asrican R, Vacanti JP, Kaban LB, et al. Reconstructing mandibular defects using autologous tissue-engineered tooth and bone constructs. *J Oral Maxillofac Surg.* 2009;**67**(2):335–47.
62. Funamoto K, Zervantonakis IK, Liu Y, Ochs CJ, Kim C, Kamm RD. A novel microfluidic platform for high-resolution imaging of a three-dimensional cell culture under a controlled hypoxic environment. *Lab Chip*. 2012;**12**(22):4855–63.
63. Keller R. Shaping the vertebrate body plan by polarized embryonic cell movements. *Science*. 2002;**298**(5600):1950–4.
64. Davies J. *Mechanisms of Morphogenesis.*Burlington: Elsevier Academic Press; 2005.
65. Rozario T, DeSimone DW. The extracellular matrix in development and morphogenesis: a dynamic view. *Dev Biol.* 2010;**341**(1):126–40.
66. Foty RA, Pfleger CM, Forgacs G, Steinberg MS. Surface tensions of embryonic tissues predict their mutual envelopment behavior. *Development.* 1996;**122**(5):1611–20.
67. Steinberg MS. Mechanism of tissue reconstruction by dissociated cells. II. Time-course of events. *Science*. 1962;**137**(3532):762–3.
68. Dean DM, Morgan JR. Cytoskeletal-mediated tension modulates the directed self-assembly of microtissues. *Tissue Eng Part A.* 2008;**14**(12):1989–97.
69. Dean DM, Rago AP, Morgan JR. Fibroblast elongation and dendritic extensions in constrained versus unconstrained microtissues. *Cell Motil Cytoskeleton*. 2009;**66**(3):129–41.
70. Krieg M, Arboleda-Estudillo Y, Puech PH, Kafer J, Graner F, Muller DJ, et al. Tensile forces govern germ-layer organization in zebrafish. *Nat Cell Biol.* 2008;**10**(4):429–36.
71. Pearson GW, Hunter T. Real-time imaging reveals that noninvasive mammary epithelial acini can contain motile cells. *J Cell Biol.* 2007;**179**(7):1555–67.
72. Wang H, Lacoche S, Huang L, Xue B, Muthuswamy SK. Rotational motion during three-dimensional morphogenesis of mammary epithelial acini relates to laminin matrix assembly. *Proc Natl Acad Sci U S A*. 2013;**110**(1):163–8.
73. Assal Y, Mie M, Kobatake E. The promotion of angiogenesis by growth factors integrated with ECM proteins through coiled-coil structures. *Biomaterials*. 2013;**34**(13):3315–23.
74. Rosines E, Sampogna RV, Johkura K, Vaughn DA, Choi Y, Sakurai H, et al. Staged in vitro reconstitution and implantation of engineered rat kidney tissue. *Proc Natl Acad Sci U S A.* 2007;**104**(52):20938–43.
75. Wolff E, Haffen K, Kieny M, Wolff E. [Tests in vitro cultures of embryonic organs in synthetic media]. *J Embryol Exp Morph.* 1953;**1**(1):55–84.
76. Willerth SM, Arendas KJ, Gottlieb DI, Sakiyama-Elbert SE. Optimization of fibrin scaffolds for differentiation of murine embryonic stem cells into neural lineage cells. *Biomaterials.* 2006;**27**(36):5990–6003.
77. Polo JM, Liu S, Figueroa ME, Kulalert W, Eminli S, Tan KY, et al. Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells. *Nat Biotechnol.* 2010;**28**(8):848–55.
78. Lee CH, Cook JL, Mendelson A, Moioli EK, Yao H, Mao JJ. Regeneration of the articular surface of the rabbit synovial joint by cell homing: a proof of concept study. *Lancet*. 2010;**376**(9739):440–8.
79. Stosich MS, Moioli EK, Wu JK, Lee CH, Rohde C, Yoursef AM, et al. Bioengineering strategies to generate vascularized soft tissue grafts with sustained shape. *Methods*. 2009;**47**(2):116–21.
80. Belema-Bedada F, Uchida S, Martire A, Kostin S, Braun T. Efficient homing of multipotent adult mesenchymal stem cells depends on FROUNT-mediated clustering of CCR2. *Cell Stem Cell.* 2008;**2**(6):566–75.