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In vitro organogenesis from pluripotent stem cells

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Pluripotent stem cells (PSCs) have the ability to spontaneously generate structured tissues in vitro reminiscent of embryonic tissue development. Recently, complex organoids such as cortical tissues, cerebral brain organoids, optical cups, intestinal tissues, and liver buds have been generated from PSCs derived from healthy individuals and patients with genetic diseases, providing powerful tools to understand morphogenesis and disease pathology. This article highlights recent advances in the state-of-art generation of organoids from PSCs, possible signaling pathways and mechanisms involved in organogenesis, and the understanding of extracellular microenvironment. Challenges involved in the organoid generation such as increasing organoid size, enhancing the tissue complexity, and improving functional maturation are also discussed.

Keywords: pluripotent stem cell, organoid, extracellular matrix, tissue development, 3-D

Abbreviations: 3-D, three-dimensional; BMP, bone morphogenetic protein; EBs, embryoid bodies; ECM, extracellular matrix; ESCs, embryonic stem cells; FGF, fibroblast growth factor; hESCs, human embryonic stem cells; hiPSCs, human induced pluripotent stem cells; hMSCs, human mesenchymal stem cells; hPSCs, human pluripotent stem cells; HUVACs, human umbilical vein endothelial cells; LB, liver bud; NOD/SCID, nonobese diabetic/severe combined immunodeficiency; PSCs, pluripotent stem cells; SFEBq, serum-free, floating EB-like quick aggregates; TGF, transforming growth factor

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Introduction

Organogenesis from pluripotent stem cells (PSCs) has been chosen by *Science*'s editors as a runner-up for 2013 Breakthrough of the Year.[1] This highlights remarkable progress achieved from extensive studies on human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), first reported in 1998 and 2007, respectively.[2,3] In theory, PSCs have unlimited proliferation capability and the ability to differentiate into nearly all types of human cells. Thus most studies have been focusing on the development and improvement of culturing of undifferentiated PSCs and directed differentiation of PSCs into specific lineages, often on adherent cultures. In recent years, the investigation on self-organization of PSC derivatives into three-dimensional (3-D) organ-like structures has also emerged.[4-6] By their unique ability to coordinate the dictatorial signals and to form tissue- and mini-organ- like structures reminiscent of cell organization in vivo, PSCs provide a powerful tool to understand the development and morphogenesis in health and disease.[7] These organ-like structures not only have the potential in regenerative medicine but can also serve as novel platforms in drug discovery to identify emerging therapeutic targets.

Three-Dimensional Aggregates of PSCs

Enhanced properties in biomedical applications

Human PSCs (hPSCs) can provide a spectrum of human cells in large quantities, which has motivated the exploration of hPSCs in drug screening and disease modeling for immediate applications. Furthermore, hiPSCs derived from patients with genetic disorders in neurological (e.g., Parkinson's, Alzheimer's), cardiovascular (e.g., long QT syndrome), and other systems can recapitulate disease-relevant phenotypes when differentiated into disease-specific cell types.[8,9] Comparative molecular and cellular studies on hiPSC-derivatives from patients and unaffected controls could lead to novel mechanistic insights into disease pathobiology.[4] Additionally, toxicity screening with hiPSC-derivatives shows that patient-specific cells (e.g., patients with long QT syndrome, familial

hypotrophic cardiomyopathy, and familial dilated cardiomyopathy) can have distinct susceptibility to cardiotoxic drugs (e.g., Verapamil, Alfuzosin) that cannot be captured when tested in animal cell line models.[9] Interestingly, 3-D organoids such as hepatocyte spheroids have shown the increased sensitivity to pharmacotoxic compounds compared with 2-D monolayer cultures.[10]

Undifferentiated PSC aggregates and embryoid bodies

Undifferentiated PSCs can self-assemble into 3-D aggregates for expansion as reported recently.[11] Histological examination of PSC aggregates revealed homogenous cell population throughout the structure, suggesting that medium composition can influence the cells in the aggregates to maintain their self-renewal and restrict the cells from the developmental path. Differentiation from PSCs can however be induced by the formation of embryoid bodies (EBs), an aggregate-like structure that contains three-germ layers.[12] Development of EBs starts with the specification of the exterior cells toward primitive endoderm, the lining of extracellular matrix (ECM) proteins collagen IV and laminin, and the interior cells surrounding a fluid-filled cavity.[13] Further differentiation from EBs leads to the formation of derivatives of the three-germ lineages. With specific inducing medium and growth factors, the EB composition can be modulated toward a particular lineage such as cardiomyocytes.[14]

Extracellular matrix

Cells in the 3-D aggregates of PSCs produce large amounts of extracellular matrices which may carry the signaling molecules and autocrine/paracrine factors (e.g., Lefty and Activin A) to modulate lineage commitment by the cells.[15,16] We and others have derived ECMs from various types of PSC aggregates at different developmental stages and demonstrated that the ECMs contain structures and signaling that can bias cell fate decisions.[17,18] The impact of PSC-derived ECMs on cellular differentiation may rely on ECM-bound growth factors such as fibroblast growth factor (FGF)-2, the biochemical composition of ECMs, and the biomechanical properties of ECMs.[19] Endogenous ECMs deposited on Matrigel have been found to support the propagation of hPSCs due to the retention of the paracrine and autocrine factors such as Gremlin and Cerberus, the antagonists of the bone morphogenetic protein (BMP) signaling.[20] However, the complex ECM microenvironment that can coax the tissue development from PSCs remains to be further elucidated.

Organoids Derived from PSCs

More complex structures other than undifferentiated PSC aggregates and the EBs with three-germ layers have been created recently to model the development of various tissue types such as cortical tissues, cerebral region of the brain, optical cups, and liver buds.[4,21,22] These complex organs contain cells that are integrated in a spatially restricted manner and provide dictatorial signals that organize the sequential developmental events.[7]

Cerebral brain organoids and optical cups (ectoderm)

Modeling brain tissues was initially done for cortical layers following formation of serum-free, floating EB-like quick aggregates (SFEBq) from hPSCs.[23,24] In the presence of FGF-2 and inhibitors of BMP, Wnt/β-catenin, and transforming growth factor (TGF)-β/activin/nodal pathways, hiPSCs can generate 3-D structures that contain polarized glia intermediate progenitors and layer-specific cortical neurons.[23] More recently, cerebral brain organoids have been formed using hiPSC-derived neural progenitors within droplets of Matrigel cultured in a bioreactor.[4] These cerebral brain organoids show regional specification of forebrain, midbrain, and hindbrain structures and the developmental changes of different regions. Furthermore, the cerebral organoids derived from a patient with microcephaly display smaller neural tissues, resembling reduced brain size in the patient.[4]

In the presence of the Wnt inhibitor and Matrigel, self-organization of hESCs using a modified SFEBq culture can lead to the formation of optical cups.[21,24,25] These optical cups contain an outer layer which develops into retinal pigment epithelium and inner neurosensory layers.[21,25] The formed neural retina spontaneously curves in an apically convex manner and forms multilayer tissues that contain rods and cones.[21] Using mouse ESCs, functional adenohypophysis can be formed through large cell-aggregation and BMP-4 treatment.[26] These adenohypophysis tissues are able to secret adrenocorticotropic hormone and rescue systemic glucocorticoid level in a hypopituitary mouse model.

Cardiac microtissues (mesoderm)

Cardiac cells derived from hPSCs can form spheres called cardiac bodies[27] or self-assemble into scaffold-free cardiac tissues.[28] These 3-D structures however lack the complex organoid structure and require further maturation. Cardiac tissue maturation may be improved by several approaches such as using 3-D scaffolds to create biomimetic cardiac wires or stimulating adult cell energy metabolism through medium formulation.[29-32] While the aggregates derived from somatic and hPSC-derived mesoderm progenitor cells have been formed,[33,34] the complex organoid structure containing multiple tissue specifications is yet to be demonstrated.

Intestine, liver, and islet organoids (endoderm)

Intestinal organoids have been derived through endoderm induction and specification of hindgut spheroids by WNT3A and FGF-4 treatment.[35] Embedding the spheroids in Matrigel allows cell maturation into intestinal organoids in a stage-specific manner similar to fetal gut development. These 3-D intestinal organoids contain all differentiated cell types of intestinal epithelium as well as villus-like domains and crypt-like progenitor niches.[35] By transient overexpression of two transcription factors, NKX2.1 and PAX8, followed by the treatment of thyroid-stimulating hormone, mouse ESCs can be induced to differentiate into thyroid follicular cells in vitro and organize into 3-D follicular structures with iodide organification activity.[36] These tissues can rescue plasma levels of thyroid hormone and promote symptomatic recovery in athyroid mice post-implantation.

The vascularized liver organoids (or liver buds: LB) can be generated by co-culturing of hiPSC-derived hepatocyte progenitors with human umbilical vein endothelial cells (HUVACs) and human

mesenchymal stem cells (hMSCs).[22] Transplantation of hiPSC-LBs in non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice leads to the generation of vascularized and functional liver that is capable of producing high levels of albumin and has the drug metabolism activity to form human-specific metabolites. Co-culture of PSC-derived progenitors with organ-matched mesenchyme has been shown to permit expansion of stage-specific progenitors along pancreatic lineages, demonstrating the context-specific coaxing of hPSC development.[37] The 3-D islet structures with β cells surrounded by glucagon-producing α cells have been generated from iPSCs, which were able to secrete insulin in response to glucose and improve blood glucose levels in hyperglycemic mice.[38] To provide 3-D microenvironment, collagen gels were also used to promote islet-like structures derived from ESCs which improved the maturation of insulin-producing cells.[39] These studies indicate that the ability of in vitro self-organization of PSC-derived pancreatic cells provides a novel approach to create functional mature tissues.[40]

Mechanisms and Methods to Form Organoids by Self-Organization

Mechanisms for organoid formation

Most organoids are formed through the process involving intrinsic tissue mechanics and the programmed internal interactions, known as self-organization. Self-organization can be classified into three categories as self-assembly (time-evolving control of relative cell positions), self-patterning (spatiotemporal control of cell status) and self-morphogenesis (deformation, local growth, and remodeling).[5,7] A "reaction-diffusion" mechanism is described as the driving force for self-patterning which involves the activation and inhibition of intrinsic signaling such as Wnt to provide spatiotemporal cues.[41] A "relaxation-expansion" model is proposed to interpret the self-morphogenesis and tissue dynamics during optic cup formation from PSCs, which involves three local rules-relaxation, apical constriction, and expansion.[42] While better understanding of self-organization needs powerful imaging tools and the knowledge in cellular mechanical properties, understanding the culture systems of the organoids formation is the pre-requisite for the mechanism study.

Appropriate starting cell population(s)

hPSC-derived progenitors are promising cell sources for organoid formation due to their ability of self-morphogenesis along a variety of developmental paths, the unlimited cell supply, and the potential for large scale or high-throughput generation.[5] Modified EB-based methods are commonly used for the formation of ectoderm organoids while the endoderm organoids usually start from monolayers of definitive endoderm progenitors. Characterization of progenitors at the intermediate stage (e.g., neuroectodermal spheres, forgut endoderm) is required and should enhance the reproducibility of organoid structure. The storage of intermediate stratified neural retina has been demonstrated to control the quality of optic cups, indicating the importance of appropriate starting cell population to form the organoids.[21]

Medium composition and different growth factors

While PSCs can spontaneously self-organize into aggregate/organoid structures, medium compositions including growth factors affect the lineage commitment and regional specification inside the organoids. For examples, the Wnt inhibitors such as IWR1-endo and DKK-1 have been applied to generate retinal progenitors or cortical tissues in the EB culture,[21,24] and epidermal growth factor, noggin, and R-spondin-1 can augment the formation of lingual organoids.[43] Cerebral organoids are simply formed in neural inductive medium containing heparin with stage-wise modification of the differentiation medium.[4] For intestinal tissues, FGF-4 and Wnt3A are used to promote the formation of hindgut spheroids.[35] These studies indicate the importance of medium composition during organoid self-organization.

3-D extracellular matrix

To form the functional organoids, 3-D matrices are usually used in the culture system. The commonly used 3-D matrices include Matrigel, collagen gel and fibrin gel probably due to their ability to actively interact with cells and to modulate cell growth and differentiation.[5,44] Progenitor cells can be embedded, encapsulated or resuspended in Matrigel to promote organoid formation.[4,35] Matrigel has also been added in the medium as the supplement to promote the neural retina formation and cardiomyocyte differentiation.[21,34] While Matrigel has been traditionally used in organotypic culture, the exact role of Matrigel and inductive factors that promote organoid formation remain to be defined. To this end, synthetic hydrogels that have defined biochemical and structural features provide an alternative matrix to Matrigel.

A system to overcome transport limitation

The size of organoids has been reported to range from 200 μm to 4 mm. With the increase in aggregate size, diffusion limitations become the limiting factor for the organoid development as seen in EBs.[45] Aggregate-size dependent diffusion limitations have been observed and modeled for EBs, which suggests that the diffusion length is about 200 μm.[45] Bioreactors (such as suspension and perfusion bioreactors) have the ability to grow large-sized organoids due to the enhanced nutrient transport, which has been demonstrated for cerebral organoids with the size about 4 mm.[4] While the bioreactors have been studied to expand and differentiate the stem cell aggregates,[46,47] how the reactor design and operation affect the organoid structure is not well understood. The vascularized structure of liver buds has also been generated in vivo through co-culture of hPSC-derived hepatic progenitors with hMSC and HUVEC cells,[22] although the potential of co-culture system to generate large-sized organoids in vitro still needs further investigation.

Perspectives and Challenges

The unique properties of PSCs to generate structured organoids provide powerful tools to study organogenesis reminiscent of in vivo tissue development, to establish high-throughput organoid assays for drug screening, and to recapitulate genetic diseases for pathobiological

study.[48] Recent advances show that a variety of mini-organoids can be formed from hPSCs including those derived from patients with genetic diseases. However, mechanisms of organogenesis are yet to be fully understood and further investigation is needed in areas including developmental signaling networks, tissue mechanics, and dynamic local interactions. It is still challenging to generate large-sized organoids with functional maturation. The regional specification of organoids has only been achieved for specific types of tissues in neuroectoderm and endoderm. To address these challenges, understanding the local extracellular matrix environment and the bioreactor systems could help accelerate the progress of hPSC-derived organoids toward clinical applications.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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