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Modeling human development in 3D culture

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Recently human embryonic stem cell research has taken on a new dimension — the third dimension. Capitalizing on increasing knowledge on directing pluripotent cells along different lineages, combined with ECM supported three-dimensional culture conditions, it has become possible to generate highly organized tissues of the central nervous system, gut, liver and kidney. Each system has been used to study different aspects of organogenesis and function including physical forces underlying optic cup morphogenesis, the function of disease related genes in progenitor cell control, as well as interaction of the generated tissues with host tissue upon transplantation. Pluripotent stem cell derived organoids represent powerful systems for the study of how cells self-organize to generate tissues with a given shape, pattern and form.

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Introduction

The development of organs and tissues from the fertilized oocyte represents a highly complex but well organized system. Studies using animal models, cell- and organotypic cultures have significantly increased our knowledge about fundamental mechanisms for organogenesis. However, continuous and detailed analysis of human development over time with its network of cell–cell interactions, cell fate decisions and differentiation steps besides inducing chemical-, genetic- and environmental factors is challenging due to limitations in human tissue availability. Thus, cell culture methodologies using *in vitro* expandable cell lines are widely used to study cellular and molecular events in lineage fate decision, differentiation and maintenance of human cells. However, as organs and tissues represent 3D objects, important aspects influencing organogenesis are missing in conventional 2D cultures and thus such approaches cannot fully recapitulate *in vivo* development and function.

Indeed, pioneering work initiated more than 30 years ago started to use collagen gels and extra cellular matrix (ECM) matrices to provide 3D scaffolds for epithelial cells of diverse origins (e.g. mammary gland/breast cancer; liver, skin/melanoma; smooth muscle; adipocytes; prostate/malignant prostate), that allowed the formation of so-called 3D spheroids. Importantly, such matrices not only provided structural support but influenced cell–cell interactions via transmembrane receptors leading to changes in cyto-skeleton organization, chromatin modulation, and gene expression [1].

In parallel, during the last three decades stem cell research has blossomed and has had a major impact on most fields of biological research including developmental-, regenerative-, or cellular studies. Specifically, the isolation and *in vitro* propagation of pluripotent embryonic stem cells (ESCs), first reported from mouse [2] and later also from human [3] blastocysts, tremendously changed the field of developmental biology by providing practically an unlimited source of cells with the potential to differentiate along the three main lineages of the body. With the advent of induced pluripotent stem cells (iPSCs) generated from somatic cells by introduction of pluripotency genes [4,5,6] a further major step for the generation of unlimited material, that can be isolated from a variety of humans and patients for studying human development and disease, are now available. Finally, defined procedures to control the differentiation of these cells along each of the three major lineages to generate cell types in two dimensional culture has been a major step forward in controlling the enormous potential of the system.

Previously, three-dimensional differentiation of PSCs was performed under relatively uncontrolled conditions to generate embryoid bodies in which cell aggregates formed tissues of several different germlayers within one aggregate. Such structures may be considered akin to teratomas that are formed when PSCs are injected subcutaneously into rodent models. This impressive ability of PSCs to self-organize into three-dimensional tissues has recently been harnessed for the formation of defined human tissues and organoids by combining 3D culture techniques with directed PSC specification protocols. Considering the possibilities to rapidly generate reporter cell lines in PSCs, as well as the ability to generate PSCs from individuals with defined genetic disorders, these

methods will revolutionize not only regenerative medicine approaches for which they are often envisioned, but will open up new horizons for studying the cell biology of human organogenesis in structures including the brain, gut, kidney and liver. Here we describe some of the major recent developments in human 3D organoid formation, including the strategies that have led to reconstitution of complex tissue architecture, as well as the application of these organoids to address previously inaccessible questions on cell differentiation and tissue morphogenesis.

Neuroectoderm: cortex and retina

The central nervous system (CNS) and in particular the brain, is one of the most accessible fate choices of ESCs. Recently the vast experience in directing pluripotent cells toward neural fates in two dimensions has been combined with three-dimensional culture in matrigel to produce various defined subregions of the developing mouse and human CNS including the cortex, retina, subpallium and adeno-hypophysis. Such organoids have been used to study the physical forces underlying retinal cup formation, species-specific dimensionality of eye tissue, as well as factors underlying progenitor cell control in the cortex.

Cortical organoids reproduce progenitor subtypes and layered structure

The cortex represents by far the biggest structure (approx. 75%) within the human brain. Its complex architecture is tightly regulated during development with the formation of distinct progenitor layers that eventually build up the six-layered adult cortex. Directed differentiation of human PSCs in two dimensional culture toward cortical neuron fate revealed a 'default' pathway of neural differentiation when PSCs are cultured under minimal conditions in the absence of exogenous morphogens and supplementation either with the bone morphogenetic protein (BMP) inhibitor Noggin [7,8] or with retinoids in combination with SMAD inhibitors [9]. Under these conditions, cortical pyramidal neuron specification followed the temporal and sequential order seen *in vivo*.

By adapting culture principles established first with mouse ESCs the Sasai group pioneered work also for early human cortical development *in vitro* [10]. Rapid aggregation of a defined number of PSCs in single wells of low attachment dishes was followed by suspension cultivation in medium containing ECM components (matrigel), yielding three dimensional neural epithelia with apical-basal polarity that further subdivided into the characteristic ventricular (VZ) and subventricular (SVZ) progenitor cell zones. Such progenitors generated cortical neurons in an appropriate temporal order with so called Cajal-Retzius-type neurons produced first forming the marginal zone basally, followed by cortical neurons constituting the cortical plate, a layer located between the SVZ and the marginal zone. Indeed, the generated neurons within these 3D cortical structures showed signs of maturation including fast-wave Ca^{2+} oscillations and formation of synapses [10,11].

Maturation of the organoids to fetal stages equivalent to the beginning of the second trimester, was achieved by culturing under increased O_2 and nutrient supplementation yielding a defined outer subventricular progenitor zone characteristic of human brains, but formation of cortical layers I–VI and mature pyramidal neurons have not yet been achieved [12•,13••]. However, disease modeling of early onset brain conditions was possible using iPSCs derived from a patient with microcephaly which generated organoids harboring reduced neuroepithelial progenitor zones with a larger proportion of differentiation, suggesting that premature differentiation causes the microcephaly phenotype, a conclusion that was supported via overexpression and knockdown of the disease-causing gene, CDK5RAP2 [13••].

Interestingly, the large cerebral organoids also included other brain regions including forebrain, hindbrain, dorsal cortex, prefrontal cortex, hippocampus, choroid plexus occipital lobe and retina, leading to the term 'mini-brains' [13••]. Previously alternate media conditions were used to produce different CNS tissue regions from mouse ESCs including subpallial patterning [14] and adeno-hypophysis [15], that might also be soon recapitulated from human PSC sources. These exciting results provide access to a broad diversity of human CNS structures for functional and morphological studies.

Eyecup morphogenesis in retinal organoids

An important highlight in self-organizing CNS tissue is the retina and fundamental insights into retinal morphogenesis have been attained through ESC-derived organoids. Slight modifications to the cortical induction protocol, particularly the use of matrigel early during controlled aggregate formation, combined with Wnt inhibition for rostralization and addition of Hh agonist, yielded retinal organoids [16••]. Such retinal epithelia expressed characteristic eyefield transcription factors like Rx and Pax6, and remarkably evaginated from the aggregates and formed optic vesicle–like structures. The retinal tissue then spontaneously invaginated forming a two-walled optic cup-like tissue with a distal neural retina portion and a proximal monolayer of retinal pigment epithelial cells. A long held concept in developmental biology was that signaling from the overlying lens epithelium directs optic cup invagination. In the first work using mouse ESCs to generate retinal organoids, Eiraku *et al.* [17] provided new insights into optic cup morphogehesis, as they showed that the organoids form optic cups in the apparent absence of lens epithelium. Additionally, the authors defined four morphological phases of optic cup morphogenesis, and using a combination of myosin light chain localization, pharmacological