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Synthetic embryology: controlling geometry to model early mammalian development

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Differentiation of embryonic stem cells *in vitro* is an important tool in dissecting and understanding the mechanisms that govern early embryologic development. In recent years, there has been considerable progress in creating organoids that model gastrulation, neurulation or organogenesis. However, one of the key challenges is reproducibility. Geometrically confining stem cell colonies considerably improves reproducibility and provides quantitative control over differentiation and tissue shape. Here, we review recent advances in controlling the two-dimensional or three-dimensional organization of cells and the effect on differentiation phenotypes. Improved methods of geometrical control will allow for an even more detailed understanding of the mechanisms underlying embryologic development and will eventually pave the way for the highly reproducible generation of specific tissue types.

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Modeling embryogenesis *in vitro*

As far back as over 2300 years ago, Aristotle made the first known written descriptions of animal embryonic development. Looking at a chick inside the egg and dissecting various mammalian embryos, he noticed that their development involves dramatic shape changes of the embryo rather than merely a continuous increase in organism size. The actual signaling pathways and morphogenetic changes were described in great detail in the previous century by manipulating the development of the frog, chick, mouse and many other animal models. Typically, these experiments involved tissue grafting experiments or applying external signals. While extremely valuable, quantitative measurements can be quite challenging in animal models. Moreover, it has become apparent that human development in many aspects follows species-specific developmental programs, often different from other mammals, such as the mouse. Therefore, in recent years, significant efforts have been devoted to designing various *in vitro* approaches to modeling embryogenesis [1–4].

Embryonic stem cells (ESCs) are an excellent proxy for the early embryo. Protocols of culturing and differentiating ES cells into a variety of tissues have seen appreciable advancements in recent years and have been used to model a number of pathways in early human development. The most common approaches to differentiating stem cells is by applying morphogens — the secreted proteins that activate a signaling program — to either disorganized 2D cell colonies on a dish or to 3D clusters of stem cells suspended in medium or embedded in Matrigel. 3D culture protocols are becoming more and more prominent, especially in creating so-called organoids, that is, differentiated 3D tissues that mimic certain aspects of adult organs in terms of tissue types and organization [4,5]. Organoids are useful in creating specific tissues or learning about signaling pathways that generate them. However, organoids do not form in a reproducible manner and rarely model the correct size and shapes seen during embryonic development [6]. Growing stem cells on pre-formed scaffolds or combining differentiation protocols with tissue origami [7] could potentially be the next strategy in designing correctly shaped tissues, however coupling differentiation with timely tissue folding would be an extremely challenging task.

Remarkably, stem cells have an innate capacity to self-organize *in vitro* in a way that mimics morphogenetic changes and cell movements during embryonic development [4]. Based on recent works, it seems that often manipulating the microenvironment of developing tissues, such as by confining cells into micropatterns or by tuning the 3D matrix can be sufficient to recapitulate complex differentiation events in early development in a highly reproducible manner. Confining cells to 2D patterns or within 3D polymeric networks does not simply impose a shape on tissues. Instead, it creates a spatially organized signaling environment, which, combined with the cell capacity to self-organize, can be a powerful tool for mimicking embryonic development in a reproducible and controlled way. Nevertheless, challenges remain, such as correctly reproducing tissue density, mechanical properties of the substrate, developmental timing, and,

importantly, achieving symmetry breaking, crucial for the formation of the body axes. Here, we highlight some recent progress in modeling of the early human and mammalian development. We focus on experiments that take advantage of controlled 2D and 3D environments and the self-organization capacity of stem cells to recreate gastrulation, neurulation, and organogenesis.

Modeling development using 2D stem cell micropatterns

Stem cells can differentiate into many different cell types, whose fate is determined depending on the applied morphogen, its concentration, time of application, cell density, and other factors. Considering the complexity of conditions that affect cell fate, there is growing interest to culture stem cells in geometrically defined colonies by seeding them on surfaces with patterned extracellular matrix (ECM) proteins (see Box 1 for technical details) [8]. Micropatterned colonies overcome the important challenge of reproducibility facing organoid research, however they also provide a quantitative platform for microscopy imaging, screening and parallelization. Interestingly, it was recently observed that cell fate can depend on the size of stem cell colonies. When presented with bone morphogenetic protein 2 and activin A, 200-μm-wide circular stem cell colonies consistently produced more endodermal genes while 1,200-μm-wide colonies produced more mesodermal genes [9]. Unconfined colonies under the same conditions would typically differentiate into a mix of mesoderm and endoderm whose ratio is difficult to reproducibly control [9]. It was therefore becoming clear that geometric control not only creates a more quantitative platform for stem cell differentiation, but that it can directly affect the fate choices of cells.

The mechanism by which geometric confinement determines cell fate was revealed in recent works where micropatterned colonies of human ESCs (hESCs) were presented with the bone morphogenetic protein 4 (BMP4). Within 48 hours, cells acquired fates of all three germ layers and trophectoderm that were radially organized forming, from edges inwards: trophectoderm, endoderm, mesoderm, and ectoderm (Figure 1) [10]. Similar to the aforementioned study [9], here small colonies mostly acquired the outermost — trophectoderm — fate, with larger colonies progressively acquiring inner fates [10]. Therefore, it seems that size *per se* is not the key factor in differentiation; rather, cell fate depends on the distance from the edge. Combined experimental and theoretical efforts demonstrated that this edge sensing is a result of (A), secretion of the BMP4 inhibitor noggin whose concentration is maximal at the colony center and (B), the localization of BMP4 receptors at the basolateral membrane of epithelial hECSs, which restricts BMP4 signaling to the outer perimeter of micropatterns [11•]. As a result, the secreted inhibitor interacts with BMP4 in a reaction-diffusion mechanism, establishing a BMP4 concentration gradient from edges inward and causing the concentric organization of germ layers. The central role of the BMP4-noggin pair was later confirmed in similar experiments, where it was suggested that a combination

Box 1 Methods of creating 2D micropatterned ESC colonies.

There are several ways of controlling the geometry of cell colonies. One common approach comprises casting a mold using an elastomeric material such as poly(dimethylsiloxane) (PDMS). The elastomer is coated with an ECM protein, such as laminin, which is then mechanically transferred like a stamp onto the dish [34] (see Box figure, *a*). Other approaches involve directly manipulating the surface by etching the surface with UV. In the depicted example (see Box figure, *b*), the laminin is deposited on the PDMS-coated surface, covered with a photomask in the shape of desired patterns, then the laminin covering the remaining surface is etched away with UV [15••].