Self-organization of neural patterns and structures in 3D culture of stem cells

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ABSTRACT

Over the last several years, much progress has been made for in vitro culture of mouse and human ES cells. Our laboratory focuses on the molecular and cellular mechanisms of neural differentiation from pluripotent cells. Pluripotent cells first become committed to the ectodermal fate and subsequently differentiate into uncommitted neuroectodermal cells. Both previous mammalian and amphibian studies on pluripotent cells have indicated that the neural fate is a sort of the basal direction of the differentiation of these cells while mesoendodermal differentiation requires extrinsic inductive signals. ES cells differentiate into neuroectodermal cells with a rostral-most character (telencephalon and hypothalamus) when they are cultured in the absence of strong patterning signals. In this talk, I first discuss this issue by referring to our recent data on the mechanism of spontaneous neural differentiation in serum-free culture of mouse ES cells. Then, I will talk about self-organization phenomena observed in 3D culture of ES cells, which lead to tissue-autonomous formation of regional structures such as layered cortical tissues. I also discuss our new attempt to monitor these in vitro morphogenetic processes by live imaging, in particular, self-organizing morphogenesis of the optic cup in three-dimensional cultures.

Keywords: stem cells, self-organization, 3D culture, emergence

1. INTRODUCTION

The behavior of stem cells, when they work as a part of society *in vitro* and *in vivo*, could be much more sophisticated than one may expect from their compositions. A typical example is recently reported self-organization of complex structures such as an optic cup in three-dimensional culture of homogenous aggregates of stem cells. In general, the spontaneous appearance of a highly ordered structure or function as a whole, which is not simply explained by the sum of the elements' complexity, is termed "emergence". In this *article*, the concept of a new research trend, which I refer to as "emergence biology", is introduced. Taking complex collective behaviors of stem cells for examples, I consider its aims and challenges in understanding the cytosystems dynamics of the cell society and discuss its applications for regenerative medicine of the next generation.

2. MULTI-CELLULAR SELF-ORGANIZATION

Animal development starts with a single fertilized egg, and through a series of numerous cell divisions, cells for the whole body are generated. During this process, "pluripotent" embryonic cells (meaning immature cells with no fate determination) differentiate into specific types such as neurons

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or muscle cells. Although this process is orchestrated by the animal's genetic program, embryonic cells that are in contact with one another communicate to decode their hidden mission, eventually forming highly ordered structures in the body.

Multicellular "self-organization" is a collective term for cell behaviors driven by local cell-cell interactions. This phenomenon allows a group of relatively homogeneous cells to spontaneously create fine-tuned structures, in the absence of external instructions. A well-known example of multicellular self-organization is callus formation in plants. When a small piece of carrot is placed under optimized culture conditions, a mass of undifferentiated cells first grows out of it with a chaotic structure. Subsequently, without external influence except for gravity, the cells form ordered structures such as a stalk and roots.

Can animal cells do this? In the classic study of sea sponges by H.V. Wilson (1907), he showed that when sponge cells are dissociated and then allowed to reaggregate, they spontaneously rebuild a basic body structure, with inner and outer cells arranged correctly in space. Townes and Holtfreter (1955) showed spontaneous reassembly of cells that were dissociated from early amphibian embryonic tissues in a tissue type-specific fashion. However, recovery of the full tissue structure has not been achieved in these approaches. Here we introduce an intriguing example of self-organization of retina from mammalian stem cells in culture.

3. LONG STANDING QUESTION

Eye development has been a favorite topic of embryologists and has attracted attention from developmental biologists for many decades. One intriguing aspect of eye development is that the retina, the main sensory organ of the eye, initially grows out of the embryonic brain (diencephalon). During early embryogenesis, the retinal neuroepithelium expands outwards (evaginates) from the diencephalic wall to form an optic vesicle. Subsequently, the distal portion of the vesicle is geometrically in contact with the surface ectoderm, and becomes fated to neural retina (sensorial tissue). In contrast, the proximal portion later differentiates into retinal pigment epithelium (RPE; supporting tissue for neural retina). The distal portion of the optic vesicle then folds inwards (invaginates), forming a two-walled cup-like structure, the optic cup, with the neural retina and RPE being the inner and outer walls, respectively. At the same time, the surface ectoderm adjacent to the retina also invaginates and develops into the lens vesicle, while the rest of the surface ectoderm near the lens becomes the cornea.

Since the time of the embryologist Hans Spemann more than a century ago, the mechanism of optic-cup formation has been a matter of debate; in particular, models have differed regarding the necessity of neighboring tissues, such as lens and cornea. Some claim that the lens physically pushes the neural retina to bend inward, while others posit that the optic cup can form without the lens tissues. A limitation of *in vivo* studies is that organogenesis takes place in such a tiny and complex environment where so many different kinds of cells are present and interact with one another.

With this in mind, my laboratory has developed a new approach using *in vitro* culture of ES cells. By recapitulating retinal development in a three-dimensional (3D) manner with minimal cell components, it has become feasible to dissect the intricate cell-cell interactions that lead to self-organization of retinal structures.

4. OPTIC CUP SELF-ORGANIZATION

In 2000, we established an efficient culture method for generating neural cells from mouse ES cells using co-culture with feeder cells. ES cells are cultured in a two-dimensional manner, as a monolayer on a culture dish. Although this method efficiently induces differentiation of ES cells into various neurons such as dopaminergic neurons, it cannot generate three-dimensional tissues.

A technical breakthrough was made in 2005 when we invented a new method using floating culture. In this method, ES cells are dissociated into single cells by a digestive enzyme that breaks cell-cell adhesion. Now suspended in culture medium, the cells are reaggregated in a small culture well containing about 0.1 ml of medium. These small aggregates of ES cells, typically 3,000 cells per well, can be efficiently induced to differentiate into neural progenitors (immature neural cells) of the rostral brain when cultured in the absence of extracellular signaling factors such as BMP, Wnt and Nodal. In 3 to 4 days, the cells in the aggregate spontaneously organize themselves into a hollow sphere consisting of a monolayered epithelium (cell sheet) of neural progenitors, called neuroepithelium. This method was named SFEBq culture (serum-free floating culture of embryoid body-like aggregate with quick reggregation).

During early embryonic development, brain regions acquire region-specific characteristics in response to their local environment. Roughly, these regions are telencephalon (cortex and basal ganglia), diencephalon (retina, hypothalamus), midbrain, hindbrain and spinal cord. These regional specificities can be given to ES cell-derived neuroepithelium by further culturing in medium containing different soluble extracellular signals. Retinal progenitors are induced to differentiate from mouse ES cells when a cocktail of extracellular matrix proteins (Matrigel) is s added to SFEBq culture.

A surprising phenomenon was observed after these spheres stayed in floating culture for several more days (Figure 2). The retinal epithelial parts in the aggregates spontaneously evaginated (protruded outwards) and formed optic vesicle-like structures. Moreover, the vesicles spontaneously deformed: the epithelial portion distal to the main body of the sphere folded inwards. This morphogenetic movement generated a brandy glass-like shape resembling the optic cup of the embryonic eye. As seen in live animals, the ES cell-derived optic cup consisted of two walls: the outer wall was RPE and the inner wall was neural retina.

Importantly, the optic cup structure formed without external positional cues. The culture was started by simply reaggregating dissociated ES cells and the cell aggregate was cultured as a floating cell mass in homogenous culture medium. The emergence of such an ordered structure from this floating mass was literally “eye-popping”. No lens or cornea formed next to the optic cup, unlike in the embryo. These observations gave a clear answer to the long-standing question of whether or not optic cup formation requires external forces from neighboring tissues such as lens cells. Retinal morphogenesis, at least in a petri dish, is a self-organizing phenomenon based on the internal program in these cell populations. In other words, retinal cells, even those derived from ES cells, can figure out what to become by working together.

5. IN VITRO FORMATION OF STRATIFIED RETINA

In mammals, the neural retina exhibits a beautiful, six-layer laminated structure, which is very important for the function and maintenance of the neural retina. As previously mentioned, the outermost layer is the photoreceptor layer, and the innermost one consists of ganglion cells that

connect the retina to the brain. Between these layers, three different categories of interneurons, called horizontal, bipolar and amacrine cells, each form their own cell layer.

When we isolated neural retina tissue from the ES cell-derived optic cup and subjected it to an additional two weeks in floating culture, the tissue grew to approximately 2 mm in diameter. Whereas the neural retina epithelium was initially single-layered, it developed a stratified structure after the two-week culture, containing all six categories of cells found in the postnatal neural retina (Figure 2). This ES cell-derived neural retina tissue was initially cultured as a floating cell mass in homogenous culture medium, indicating that the multi-layer formation is another self-organizing phenomenon. The neural retinal progenitor cells therefore contain an internal program that guides what kinds of cells to make and how to arrange them in 3D space.

The self-organizing formation of the optic cup and laminated neural retina demonstrate the presence of "latent intrinsic order" in retinal progentior cells, which emerges when cells work together via cell-cell interactions under permissive conditions. The free-floating culture with relatively simple medium that we used presumably allowed cells to behave according to their intrinsic program, instead of responding to external signals. Local intercellular communications that enable this amazing collective behavior are likely to be delicate, requiring a reasonably "quiet" environment in order to be heard.

6. MECHANISTIC CONSIDERATIONS

There are many questions left unanswered regarding the self-organization of optic cup from ES cells. For instance, how do cells generate a patterned structure from a cell aggregate with no pattern? Spontaneous emergence of a pattern or polarity from homogenous matter is generally called "symmetry breaking". Symmetry breaking is known to occur in many aspects of embryonic development. If it were not for symmetry breaking, repeated cell divisions of the fertilized egg would create just a mass of cells rather than a patterned body. Our ES cell culture for self-organization may be an ideal experimental platform for understanding the profound mechanism of symmetry breaking in mammalian embryogenesis, which remains largely elusive.

Another big question is how the shape of the optic cup is programmed internally in the retinal epithelium, which is initially just a sheet of cells. In general, the strain of tissue is controlled by two local factors: local force and tissue stiffness. By measuring the internal force direction and tissue stiffness in different domains of retinal epithelium during optic cup formation *in vitro*, we found that only three local mechanical rules sufficiently drive this complex morphogenesis. These are 1) reduced stiffness in neural retina, 2) wedge-shaping of cells at the junction of neural retina and RPE and 3) rapid proliferation of the invaginating neural retina. A computer simulation also confirmed that these three mechanical changes could cause optic cup morphogenesis in retinal epithelium. This provided support for the idea that the self-driven optic cup shaping depends on a relatively simple internal program governing local tissue mechanics.

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