Review Article

Neural Commitment of Embryonic Stem Cells through the Formation of Embryoid Bodies (EBs)

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Abstract

An embryonic stem cell (ESC) is a good tool to generate neurons in vitro and can be used to mimic neural development in vivo. It has been widely used in research to examine the role of cell signalling during neuronal development, test the effects of drugs on neurons, and generate a large population of functional neurons. So far, a number of protocols have been established to promote the differentiation of ESCs, such as direct and indirect differentiation. One of the widely used protocols to generate neurons is through the spontaneous formation of multicellular aggregates known as embryonic bodies (EBs). However, for some, it is not clear why EB protocol could be the protocol of choice. EB also is known to mimic an early embryo; hence, knowing the similarities between EB and an early embryo is essential, particularly the information on the players that promote the formation of EBs or the aggregation of ESCs. This review paper focuses on these issues and discusses further the generation of neural cells from EBs using a well-known protocol, the 4−/4+ protocol.

Keywords: embryonic stem cells, embryoid bodies, neural differentiation, retinoic acid, cell aggregation

Introduction

One of the unique characteristics of embryonic stem cells (ESCs) is that they can differentiate into the three primary germ layer derived cells (1,2). This together with another characteristic, their ability to self-divide makes ESCs a reliable tool to generate a large number of functional cells and a good model to study the development of early embryos. However, the balance between self-renewal and differentiation of ESCs is controlled by a complex network of genes and signalling pathways. This complex network, at a certain level, complicates the control of the differentiation process and thus, generates a large number of specific cell types. Many protocols have been established to differentiate ESCs into specific cell types such as neurons, islet cells, cardiomyocytes, and so on (3,4). Although the supplements used and the procedures of these protocols are very different from each other, in general, there are two types of differentiation protocols; indirect and direct differentiation. Different from direct differentiation, in which differentiation of ESCs is "directed" to certain cell types by using specific exogenous chemicals and factors, indirect differentiation is more dependent on endogenous factors and the specific signalling pathways involved. Indirect differentiation is also called spontaneous differentiation. Spontaneous differentiation means to withdraw the pluripotent-dependent factors (such as leukemia inhibitory factor (LIF) and feeder layers), therefore making the balance to favour differentiation. The spontaneous differentiation of ESCs is demonstrated through the formation of embryoid bodies (EBs). EBs are multicellular 3D aggregates that contain the cells of the three primary germ layers. EB is widely used in examining mammalian development in vitro (5–7).

In contrast, directed differentiation can occur without going through the formation of EBs but directly generate the specific cells from ESCs.

Instead of the formation of EBs, some growth factors and chemicals such as fibroblast growth factor (FGF) (8), noggin (9), N2, and B27 (8) are added to trigger the neural differentiation of ESCs as monolayer-adherent cells. In addition, culturing the ESCs in low density under defined conditions (without serum and feeder layer) could also trigger neural differentiation (9).

Although both direct and spontaneous differentiation methods are efficient and widely used, an advantage of spontaneous differentiation through the formation of EBs is that it can be used as one of the important "golden rules" to check the pluripotency of any pluripotent stem cells (including iPSCs and ESCs). For example, human amniotic fluid stem cells (AFSCs) have been found to form EBs spontaneously, in which the study provides evidence of pluripotency in AFSCs (10). Hence, knowing the essential information about the formation of EBs might be useful to those who have decided on checking the pluripotency of stem cells or generating neural cells under a condition mimicking the embryo development in vivo.

This review focuses on applying a spontaneous differentiation protocol to generate neural lineage from ESCs. It starts with the introduction of EBs and summarises the techniques used in generating EBs, followed by the introduction of the players behind the formation of EBs, particularly those that promote the neural differentiation. The similarities between EBs and early embryos are also discussed in this review.

## Embryoid Body (EB) and its Formation

EBs are made from ESCs; each EB is a multicellular 3D aggregate that contains partly differentiated ESCs and a cavity caused by cell death (7) (Figure 1). During spontaneous differentiation, the cells inside the EB keep dividing and interacting with each other, which then lead to the generation of the three primary germ layer–derived cells. The formation of EBs starts from the aggregation of ESCs. In this process, the balance between non differentiated and differentiated ESCs can be broken by withdrawing the pluripotent-related factors. Differentiation of ESCs through the formation of EBs generally follows three steps: (1) culturing of ESCs in suspension without LIF for mouse ESCs or without a feeder layer for human ESCs, (2) spontaneous differentiation of EBs in a non-coated dish, and (3) directed differentiation of mature EBs into specific cell lineages with the addition of growth factors/chemicals. At the first step of EB formation, the density of ESCs is

crucial for the quality of EBs (11). The quality of EBs also influences the differentiation efficiency of ESCs toward specific cell lineages (11). Simple ways to judge the quality of EBs normally depend on the size/shape and morphology of EBs during spontaneous differentiation (11,12). The size of EBs has been found to influence the differentiation efficiency of human and mouse ESCs (12,13). For example, a smaller EB around 150–300 μm in diameter is suitable for endothelial cell differentiation, but bigger EBs around 450 μm in diameter can promote the cardiogenesis of ESCs (14). In addition, the shape of EBs is also affected by the environment surrounding the EBs such as the cell signals, extracellular matrix, and the material of the plate/dish (15).

In order to obtain good-quality EBs, the spontaneous differentiation method has been optimized. Optimized protocols have been established to control the size and the shape of EBs. These include the suspension culture in the petri dish, hanging drop, and microwells/capsule methods (Figure 2).

Suspension culture in the petri dish

The suspension culture in the petri dish (Figure 2a) is the simplest method to make a large amount of EBs at the same time. Generally, ESCs are harvested from a feeder layer and counted and suspended inside a non-coated petri dish to allow them to aggregate and form EBs. This method can generate a large number of EBs in one petri dish, but the size/shape of each EB cannot be controlled (11,12). The material of a petri dish is also crucial for the formation of an EB. Only a non-surface-treated petri dish (normally a bacteriological-grade petri dish) can be used in this process to prevent uncontrollable attachment of ESCs to the bottom of the dish instead of just the cell-to-cell attachment. The appropriate materials of the petri dish/well are also important for the culturing of EBs. Inappropriate dishes/wells
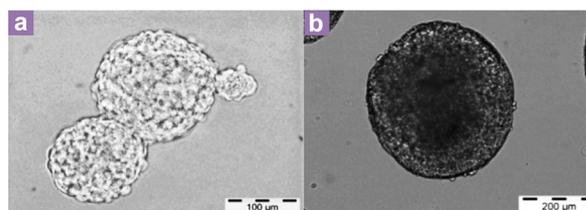
Figure 1: The phase contrast images of ESC aggregates and EBs. (a) shows the aggregates of ESCs and (b) shows the morphology of EBs.

might cause the attachment of EBs and reduce the efficiency of differentiation (16,17). Petri dishes made of different materials, such as polystyrene (PS), polypropylene (PP), and phosphorylcholine were tested during the formation of EBs. PP has been found to be better than other materials for making EBs (16). In addition, petri dishes coated with chemicals may also enhance the formation of EBs and reduce the attachment. For example, a 2-methacryloyloxyethyl phosphorylcholine–coated surface has been found to protect cells from attaching to the bottom of dishes, and silicon-coated glass petri dishes are good for the formation of EBs (17,18). Therefore, for the formation of EBs, the suitable petri dish or the specific type of dish is very important for the differentiation efficiency of EBs.

Hanging drop

Hanging drop is a method (Figure 2b) to generate homogenous-size EBs by suspending single cells in drops, which hang on the cover of a petri dish, and each hanging drop contains a certain number of ESCs. The ESCs aggregate at the bottom of droplets upon being affected by gravity (19). The hanging drop method successfully prevents EBs from attaching to the surface of the container and controls the size and the shape of EBs. However, this method has some disadvantages. First, it cannot produce a high number of EBs at the same time because each drop only contains a few EBs. Second, changing the medium and treatment of EBs is very difficult to carry out.

Microwell/microcapsule

Microwell/capsule methods (Figure 2c) were adopted to control the size, shape, and homogeneity of EBs. Round-bottom 96-well plates have been used to form EBs because these plates were found to be better than the flat-bottom 96-well plates for the formation of EBs (17). Polyacrylamide hydrogel made microwells good for culturing EBs in 3D. Polyacrylamide hydrogel microwells are stable in an EB medium, and the surface of polyacrylamide hydrogel can prevent ESCs from attaching to the surface of the well. In addition, they can create a hydrated niche condition for ESC differentiation (20). The size of EBs generated in the microwell method depends on the initial cell density in each well as well as the dimension of the wells. Normally, the optimal size of EBs ranges from 100 to 500 μm (14,20,21). Compared with the hanging drop method, changing the medium, treatment, and collection of EBs is easier done with the microwell method.

Moreover, EBs trapped in hydrogel microwells can be protected from the stresses of fluid flow (15).

The players behind aggregation of embryonic stem cells

There are several important factors that regulate attachment of ESCs and the formation of EBs. At the early stage of EB formation, ESCs cultured in suspension attach to each other and form ESC aggregates (Figure 3a). The surface of the EB becomes smoother and rounder compared to early cell aggregates because of the differentiation of ESCs on the outer layer of the EB (Figure 3b) (7,22). However, not all ESC aggregates will differentiate into mature EBs; a report found that culturing mouse ESC aggregates under microgravity conditions has managed to maintain the pluripotency of ESCs even in the absence of LIF (23). Therefore, finding the best players behind the differentiation of ESCs is important in order to understand more about neural development and generate more neuronal cells (Figure 4).

Cell signalling pathway and growth factors

Some factors that are used to maintain the undifferentiated status of ESCs, such as LIF for mouse ESCs (mESCs) and mouse embryonic fibroblast (MEF) feeder layer for human ESCs (hESCs), have to be removed during the formation of EBs (24–26). LIF/Stat3 is one of the main LIF pathways that have been found to regulate pluripotency of mESCs. Pluripotent-related genes such as *Nanog, Gdf3, Rex1, Rest, Socs3, CD9,* and *Tdgf1* have been found to be upregulated by
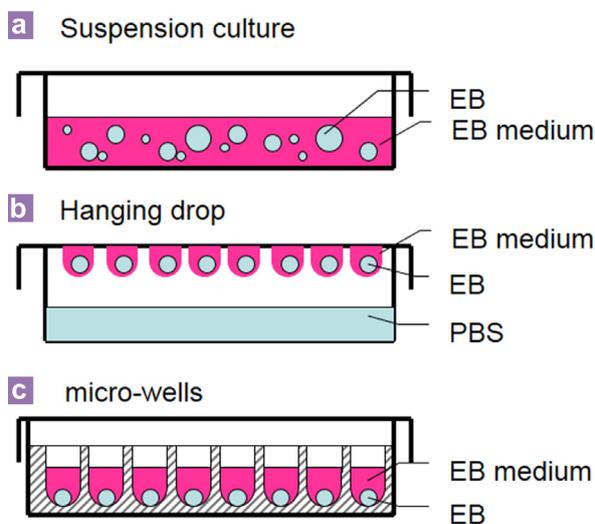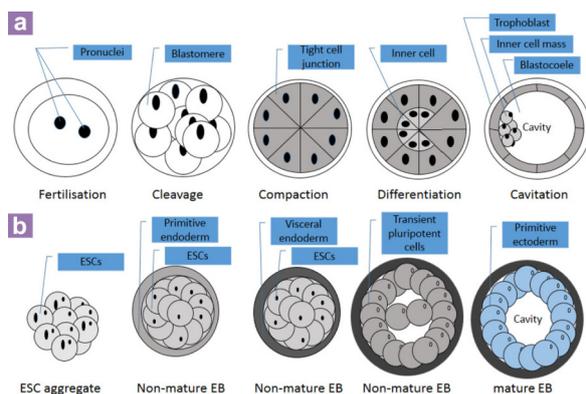

Figure 2: Methods to form embryoid bodies.

the LIF/Stat3 signalling pathway in mESCs, but *Brachyury, Eomes, Foxa2, Gata6,* and *Lhx1* were downregulated (27). Different from mESCs, a LIF/Stat3 signalling pathway is not sufficient for maintaining the self-renewal and pluripotency of hESCs (2,28).

The Wnt signalling pathway, which is known to regulate the self-renewal and differentiation of ESCs, has also been found to be crucial for the development of the central nervous system (CNS). Nordin et al. (2008) found that *Wnt3, 5b, 6, 7a, 7b, 8a, 9a, 10b,* and *16* were expressed in ESCs, and upon screening 19 mouse Wnt genes during neural differentiation of mESCs through the formation of *EBs, Wnt3, 3a, 5a, 5b, 7b, 8a, 9b,* and *10b* were found to be expressed in early EBs, but the expression of *Wnt6, 7a, 7b,* and *8a* decreased dramatically during the spontaneous differentiation of mESCs and the formation of EBs (29). Wnt signalling pathways also regulate
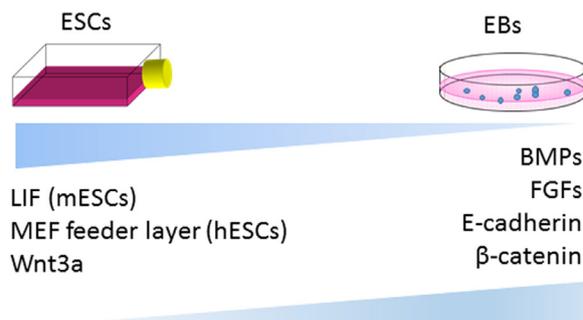

Figure 3: The development of an early embryo (a) and the formation of an EB in vitro (b). (Images are adapted and modified from: http://en.wikipedia.org/wiki/File: HumanEmbryogenesis.svg, September 2013, 33 and 7).

Figure 4: The players behind the formation of EBs.

the proliferation and differentiation of hESCs. Wnt3a was found to improve the self-renewal and "stemness" of hESCs, but the activity of Wnt/β-catenin signalling only increased during differentiation of hESCs (30).

Bone morphogenetic proteins (BMPs) are another important factor for early differentiation of an embryo in vivo. Different from the function that Wnt signalling plays during neural differentiation, BMP signals can promote endodermal and mesodermal differentiation (31–35). Together with fibroblast growth factors (FGFs), BMPs are able to promote the primitive endodermal differentiation both in vivo and in vitro (7,36). Moreover, FGFs and BMPs regulate the formation of visceral endoderm, which provides an important source of cellular signals during the gastrulation of an embryo and cavitation of EB (7,37–40).

The changes in the expression of certain genes happen rapidly during the spontaneous differentiation of mESCs. Research on the early differentiation process of mESCs found that within the first 12 hours after spontaneous differentiation, *Pim1, Pim3, SOCS3, Anxa3, Mras* (signalling-related proteins), *Fblim1, Vim, Tagln, Mapt, Brca2, Bhlbh2, Bcl3, Klf4, Klf5,* and *Nrob1* (nuclear proteins) were downregulated; meanwhile, *TAPP2* (signalling-related protein), *Wdr1, Arpc5, and Myl9* (cytoskeleton-related proteins) and *Smn1, Phf21a, Myb,* and *Otx2* (nuclear proteins) were upregulated (41).

Cell adhesion factors

Some cell surface proteins called adhesion molecules regulate the cell-cell adhesion of ESCs. These factors play important roles in the aggregation of ESCs and differentiation of EBs (42). E-cadherin is one of the cell-cell adhesion molecules; it regulates the aggregation of hESCs and mESCs (42–44). The cell-cell adhesion defect was found to increase the proportion of single cells and decrease the size of EBs (42). An increasing number of studies have suggested that β-catenin may act as a cell-cell adhesion factor during the formation of EBs (45–47). Previous studies found that β-catenin is crucial for the differentiation of neuroepithelia, the formation of telencephalon, and the development of the nervous system in vivo (45,47,48).
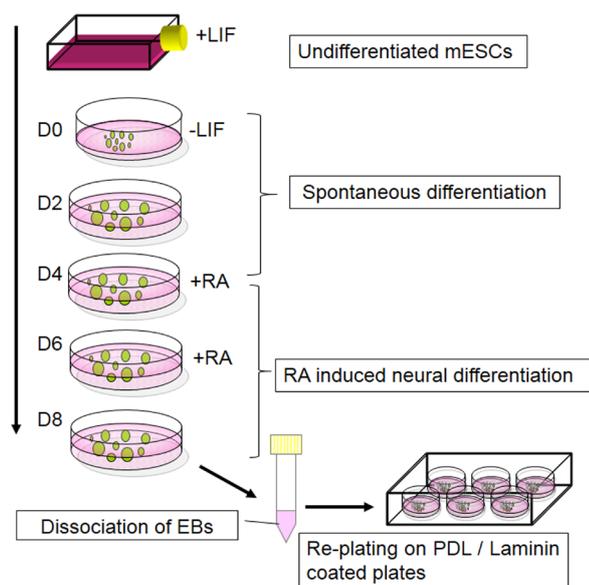
Embryoid Body versus Early Embryo: The Similarities

At around embryonic day 6.5 (E6.5) of a mouse embryo, the inner cell mass inside the

blastocyst will differentiate into the epiblast and subsequently into the primitive ectoderm (39, 49). The primitive ectoderm of an early embryo further develops into mesoderm, ectoderm, and endoderm during gastrulation (50). Similar to the gastrulation process in vivo, inner cell mass-derived ESCs are able to differentiate into the three germ layers through a gastrulation-like process in vitro (Figure 3a) (7). In EBs, the formation of a primitive ectoderm is followed by the visceral endoderm on the surface of EBs (Figure 3b) (7, 49). The formation of epiblast cells and visceral endoderm can be found in both embryos and EBs (7). Although both EBs and embryo possess the same ability to differentiate into the three germ layers, primordial germ cells (PGCs), which further develop into sperm and egg, can only be derived from the embryo before the gastrulation. Moreover, because of the same structure of the cavity in both the early embryo and EBs, the ESC-derived EBs have been used as an in vitro model to examine the process of cavitation during embryo development (39) (Figure 5).

Embryoid Body Protocol of Neural Differentiation (4–/4+ protocol)

The 4–/4+ protocol is one of the widely used protocols to differentiate ESCs into neurons through the formation of EBs (52). To induce differentiation of ESCs by the 4–/4+ protocol, spontaneous differentiation of EBs is essential during the first four days of EB culture. During this period, EBs develop into mature EBs, where they are exposed to all-trans retinoic acid (RA) for another four days to promote the differentiation toward neural lineages (Figure 5). This method has been successfully used in neural differentiations of mESCs and hESCs (53,54).

RA has been found to be expressed in early embryos as a morphogen that regulates the development of the neural tube in vivo and triggers neural differentiation during the early development of the CNS (55). When RA is present, it binds to the receptor complex, which is composed of retinoic acid receptors (RARs) and retinoid X receptors (RXRs) in the cell nucleus, and activates the transcription of target genes, including *RARs, Hox genes, HNF-3α,* and *Cdx1* (56,57). The RA signalling pathway regulates anteroposterior patterning of the CNS and the migration of hindbrain neural crest through the expression of *Hox* genes (58–60). It can also be used as a supplement to trigger neuroectodermal differentiation in vitro (52). Previous research found that the neural differentiation of EBs could be increased up to six times compared with spontaneous differentiation without in RA (61), suggesting that the effect of RA is efficient during neural differentiation in vitro. However, RA not only promotes neural differentiation of the CNS but also differentiation toward other organs, such as skeleton, forelimb, heart, somites, eyes, pancreas, and lungs (62–65). The effect of RA is concentration-dependent during the embryonic development in vivo (66). Therefore, the concentration of RA is crucial for EB differentiation. Studies found that high concentrations of RA (10^{-7} M) have led to neural differentiation (56), while low concentrations of RA (10^{-8} M) triggered differentiation toward vascular smooth muscle (68). Lower concentrations of RA (10^{-9}–10^{-8} M) can trigger differentiation toward cardiogenic cells (68). Furthermore, for the neural differentiation of EBs, a higher concentration of RA (around 2 × 10^{-6} M) is better than a low concentration of RA in generating more postmitotic neurons and glia, but a lower concentration of RA (around 2 × 10^{-8} M) can increase the population of neural progenitor cells (69).

The EB protocol can be used to differentiate ESCs into motor neurons and oligodendrocytes of the ventricular region and dorsalises neural progenitors (69). A study found that the appearance of neural precursor cells (NPCs) in day 6 EBs and the population of NPCs reached the peak around day 8 (29).

Although the EB-based 4–/4+ protocol is

Figure 5: 4-/4+ protocol.

widely used, some disadvantages were observed during RA-induced neural differentiation (69–71). Studies found that RA treatment causes the caudalisation of the neural tube and reduces cell proliferation of the chick embryo (69,70). In some cases, RA-treated progenitors show a limited capacity to differentiate after transplanting into the embryonic chick neural tube (71).

Besides the EB-based, RA-induced neural differentiation (4–/4+) protocol, there are some other chemicals and cell signalling proteins that can be used to trigger the neural differentiation of EBs. Cell signalling pathways such as FGF, Wnts, BMP, and Sonic hedgehog (SHH), which are involved in neural differentiation of ESCs (69,72–74), are potential candidates to be used to establish more therapeutic-promising neural differentiation protocols in the future.

Conclusion

In this review, we focused on the mechanism of the spontaneous differentiation method, which can be used to differentiate ESCs through the formation of EBs toward neural lineages. Understanding the mechanisms of EB formation processes could allow us to use them as a suitable tool to study embryo development and neurogenesis in vitro. A number of EB-based protocols have been established to differentiate ESCs into different cell types, but the basic principles behind these methods are more or less similar. Because of the similarities of EB and pregastrulation embryo, spontaneous differentiation (EB method) is widely used in examining the effect of the cell signalling pathway and the effect of chemicals during embryogenesis. The presence of the three primary germ layer–derived cells in EBs under certain conditions has made it a valuable tool in providing a standard mean to examine the pluripotency of stem cells or generate specific cell types from certain pluripotent stem cells (such as ESCs and iPSCs). In contrast, directed differentiation via monolayer-adherent cells is more straightforward and generally faster in getting certain cell types than spontaneous differentiation. However, the method does not really mimic the in vivo embryonic development. The 4–/4+ neural differentiation protocol was discussed in the last part of this review as an example of how this protocol can be used to produce specific cell lineages (neural cells) from EBs. Hopefully, this review may provide some useful information for those who are interested in cell signalling pathways and mammalian development.

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