Embyoid body formation from embryonic and induced pluripotent stem cells: Benefits of bioreactors

Sasitorn Rungarunlert, Mongkol Techakumphu, Melinda K Pirity, Andras Dinnyes

Abstract
Embryonic stem (ES) cells have the ability to differentiate into all germ layers, holding great promise not only for a model of early embryonic development but also for a robust cell source for cell-replacement therapies and for drug screening. Embryoid body (EB) formation from ES cells is a common method for producing different cell lineages for further applications. However, conventional techniques such as hanging drop or static suspension culture are either inherently incapable of large scale production or exhibit limited control over cell aggregation during EB formation and subsequent EB aggregation. For standardized mass EB production, a well defined scale-up platform is necessary. Recently, novel scenario methods of EB formation in hydrodynamic conditions created by bioreactor culture systems using stirred suspension systems (spinner flasks), rotating cell culture system and rotary orbital culture have allowed large-scale EB formation. Their use allows for continuous monitoring and control of the physical and chemical environment which is difficult to achieve by traditional methods. This review summarizes the current state of production of EBs derived from pluripotent cells in various culture systems. Furthermore, an overview of high quality EB formation strategies coupled with systems for in vitro differentiation into various cell types to be applied in cell replacement therapy is provided in this review.

Recently, new insights in induced pluripotent stem (iPS) cell technology showed that differentiation and lineage commitment are not irreversible processes and this has opened new avenues in stem cell research. These cells are equivalent to ES cells in terms of both self-renewal and differentiation capacity. Hence, culture systems for expansion and differentiation of iPS cells can also apply methodologies developed with ES cells, although direct evidence of their use for iPS cells is still limited.

Key words: Embryoid body; Embryonic stem cells; Induced pluripotent stem cells; Bioreactors; Differentiation

Peer reviewer: Takashi Tada, Professor, Stem Cell Engineering, Institute for Frontier Medical Sciences, Kyoto University, 53 Kawahara-cho, Shogoin, Sakyoku, Kyoto 606-8507, Japan

© 2009 Baishideng. All rights reserved.
pluripotent stem cells: Benefits of bioreactors. *World J Stem Cells* 2009; 1(1): 11-21 Available from: URL: http://www.wjgnet.com/1948-0210/full/v1/i1/11.htm DOI: http://dx.doi.org/10.4252/wjsc.v1.i1.11

---

**INTRODUCTION**

Embryonic stem (ES) cells are capable of unlimited self-renewal *in vitro* and differentiate into cells constituting all three somatic germ layers. ES cells were first isolated from the inner cell mass of mouse blastocyst stage embryos,[1,2] subsequently, followed by the derivation of non-human primate and human ES cell lines.[3,4] Currently, an alternative method has derived pluripotent cells by retroviral transduction of a combination of four transcription factors, Oct4, Sox2, C-myc and Klf4 into somatic cells; known as “induced pluripotent stem (iPS) cells”.[5,6] These cells are equivalent with ES cells in terms of both self-renewal and differentiation capacity.[7,8] The unique ability of pluripotent cells to generate a vast range of different cells makes both ES and iPS cells suitable for various cell transplantation, tissue engineering and drug testing applications. Efficient and controlled means of directing ES or iPS cell differentiation is crucial for the development of cell replacement therapies.[9,10]

To realise the therapeutic potential of ES cells, it is essential to regulate their differentiation in a reproducible manner. Differentiation of ES cells is performed in two main ways; either by direct differentiation from pluripotent cells or through the formation of cell aggregates in non-adherent spheroids, called embryoid bodies (EBs).[11,12] The molecular and cellular morphogenic signals and events within EBs recapitulate numerous aspects of the embryo development and result in differentiation to cells of three embryonic germ layers (endoderm, mesoderm, and ectoderm lineages), similar to gastrulation of an epiblast-stage embryo *in vivo*.[3] The precise number and spatial coordination of the various cell-cell interactions involved in EB formation are considered to influence the course of ES cell differentiation and, as a result, the control of cell number, size of EBs and quality of EB formation are important step directed differentiation strategies.[14,15]

Methods of inducing EB formation are based on preventing ES cells from attaching to the surfaces of culture vessels, thus allowing the suspended ES cells to aggregate and form EBs. Standard methods of achieving EBs are *via* hanging drop and in static suspension culture to allow small scale formation of aggregates. These culture systems maintain a balance between ES cell aggregation essential for EB formation and prevention of EB agglomeration.[16] Even though hanging drop method is commonly used to prepare uniform-sized EBs (see details below), this method has disadvantages in the mass preparation of EBs due to its labor-intensive procedure, which hinders the use of differentiated ES cells for therapeutic application.[17] Mass EB production is easier from static suspension culture in which ES cells are suspended in a static Petri-dish. One drawback of this method, however, is that the EBs often fuse together to form large aggregates. This has negative effects on cell proliferation and differentiation, as well as causing extensive cell death. Hence, these methods are restricted as far as industrial applications are concerned because of their complication and difficult manageability.[18]

Recently, novel bioreactors for large-scale production of ES-derived cells have been developed. A bioreactor is often defined as a device in which biological processes (cell expansion, differentiation or tissue formation on biomaterial scaffolds) occur in a tightly controlled environment *in vitro*, including the exchange of oxygen, nutrients and metabolites.[19] There are several types of bioreactors. For example, stirred suspension cultures (spinner flasks) have been successfully employed in some studies of mass scale production of ES-derived cells.[20,21] Conventional stirrer vessels may have the disadvantage of generating shear forces and, although manageable, these forces still can damage the cells.[22] Another bioreactor that allows agglomeration-free EB formation is the rotating cell culture system (RCCS) developed by the US National Aeronautics and Space Administration (NASA). This system is characterized by EB immobility in space, due to an extremely low fluid shear stress and oxygenation by diffusion.[23] EBs produced by bioreactors were more uniform in size and had less necrotic centers in comparison to static suspension culture. Furthermore, bioreactors can be also used for culturing iPS cells, which is expected to become a main further application of mass EB production in the near future. This review is focused on EB production in different systems, provides data on a number of existing bioreactors in comparison to conventional methods (hanging drop and static suspension culture) and describes differentiation of end-product EBs towards specific lineages.

**METHODS FOR CULTURING EMBRYOID BODIES**

At the present time, no universally accepted standard exists for measurement of EB formation although characteristics such as EB size, shape, homogeneity and the quality of EB formation, ratio of apoptotic and viable cell are typically used as benchmarks for evaluation.[24] There are several methods to generate EB formation, as schematically shown in Figure 1. A summary of all of the important methods (described below) is presented in Table 1. Traditionally the most common EB culture methods, such as hanging drop method and static suspension culture, were used for inducing differentiation.

**Hanging drop method**

The hanging drop method (Figure 1A) provides uniform sizes of EBs by dispensing equal numbers of ES cells in physically separated droplets of media suspended from the lid of a Petri-dish. This method offers a similar environment for forming individual EBs within each drop *via*
| Cell culture methods | Benefit (a) | Detriment (b) | Propose (c) | Yield (d) | Note (e) | Ref. (f) |
|----------------------|------------|--------------|-------------|-----------|----------|---------|
| 1 Hanging drop       | x          | x x x        | Differentiation into three germ layers | ND        | Using mES cells | [26] |
| 2 Static suspension  |            |              | Differentiation into three germ layers and neural lineage | ND        | Using mES cells | [26,31] |
| culture              |            |              | Differentiation into hematopoietic lineage | ND        | Using mES cells | [26] |
| 3 Entrapment of ES   | x          | x x x        | Differentiation into cardiac and neural lineage | 94% of wells have a single EB with diameter of 415 microns | Using polyvinyl carbonate PCR plate without coating reagents | [17] |
| cells (methylcellulose)|          |              | Differentiation into cardiac lineage | EB formed MPC and CS was increased cardiac differentiation | ND | Comparison of EB formation derived various type of 96 well plate; PS and PS coated with MPC | [28] |
| 4 Multitwell/        |            |              | Differentiation into hematopoietic lineage | Single EBs were achieved from PC surface but not from PS surface | Comparison of EB formation derived various type of 96 well plate; PS and PS coated with MPC | [28] |
| microfabrication     |            |              | Differentiation into hematopoietic lineage | > 90% EB formation was produced in a single run | Comparison of EB formation derived various type of 96 well plate; PS and PS coated with MPC | [28] |
| 4.1 Round bottomed,  | x          | x x x        | Differentiation into cardiac lineage | Single EB achieved from PS coated with MPC was near 100% | Force aggregation by using centrifugation; Using mES cells | [28] |
| low attachment, 96   |            |              | Differentiation into cardiac lineage | EB formed MPC and CS was increased cardiac differentiation | ND | Force aggregation by using centrifugation; Using mES cells | [28] |
| well plate           |            |              | Differentiation into hematopoietic lineage | Single EBs were achieved from PC surface but not from PS surface | Comparison of EB formation derived various type of 96 well plate; PS and PS coated with MPC | [28] |
| 4.2 Low adherence, 96 | x          | x x x        | Differentiation into cardiac lineage | Single EB achieved from PS coated with MPC was near 100% | Force aggregation by using centrifugation; Using mES cells | [28] |
| well plate coated with |            |              | Differentiation into cardiac lineage | Single EB achieved from PS coated with MPC was near 100% | Force aggregation by using centrifugation; Using mES cells | [28] |
| MPC or CS            |            |              | Differentiation into hematopoietic lineage | Single EBs were achieved from PC surface but not from PS surface | Comparison of EB formation derived various type of 96 well plate; PS and PS coated with MPC | [28] |
| 4.3 Round bottomed,  | x          | x x x        | Differentiation into hematopoietic lineage | Single EBs were achieved from PC surface but not from PS surface | Comparison of EB formation derived various type of 96 well plate; PS and PS coated with MPC | [28] |
| low attachment, 96   |            |              | Differentiation into hematopoietic lineage | Single EBs were achieved from PC surface but not from PS surface | Comparison of EB formation derived various type of 96 well plate; PS and PS coated with MPC | [28] |
| well plate           |            |              | Differentiation into hematopoietic lineage | Single EBs were achieved from PC surface but not from PS surface | Comparison of EB formation derived various type of 96 well plate; PS and PS coated with MPC | [28] |
| 4.4 Round bottomed,  | x          | x x x        | Differentiation into cardiac lineage | Single EB achieved from PS coated with MPC was near 100% | Force aggregation by using centrifugation; Using mES cells | [28] |
| low attachment, 96   |            |              | Differentiation into hematopoietic lineage | Single EB achieved from PS coated with MPC was near 100% | Force aggregation by using centrifugation; Using mES cells | [28] |
| well plate           |            |              | Differentiation into hematopoietic lineage | Single EB achieved from PS coated with MPC was near 100% | Force aggregation by using centrifugation; Using mES cells | [28] |
| 4.6 V bottomed, 96   | x          | x x x        | Differentiation into cardiac lineage | > 90% EB formation was produced in a single run | Using mES cells | [28] |
| well plate           |            |              | Differentiation into cardiac lineage | > 90% EB formation was produced in a single run | Using mES cells | [28] |
| 5 Bioreactor         |            |              | Differentiation into cardiac lineage | > 90% EB formation was produced in a single run | Using mES cells | [28] |
| 5.1 A 2-L controlled | x          | x x x x x    | Differentiation into cardiac lineage | 4.6 × 10⁶ of cardiomyocytes were produced in a single run | Using MHC-neo ES cells | [31] |
| spinner flask        |            |              | Expansion and differentiation into three germ layers | EB formed MPC and CS was increased cardiac differentiation | ND | Comparison of stirred and static suspension culture | [28] |
| 5.2 Stirred          | x          | x x x x x    | Differentiation into cardiac lineage | 4.6 × 10⁶ of cardiomyocytes were produced in a single run | Using MHC-neo ES cells | [31] |
|                      |            |              | Expansion and differentiation into three germ layers | EB formed MPC and CS was increased cardiac differentiation | ND | Comparison of stirred and static suspension culture | [28] |
| 5.3 Stirred          | x          | x x x x x    | Differentiation into vascular lineage | 4.6 × 10⁶ of cardiomyocytes were produced in a single run | Using MHC-neo ES cells | [31] |
|                      |            |              | Expansion and differentiation into vascular lineage | 4.6 × 10⁶ of cardiomyocytes were produced in a single run | Using MHC-neo ES cells | [31] |
| 5.4 Stirred          | x          | x x x x x    | Differentiation into cardiac lineage | 4.6 × 10⁶ of cardiomyocytes were produced in a single run | Using MHC-neo ES cells | [31] |
| 5.5 Stirred          | x          | x x x x x    | Differentiation into cardiac lineage | 4.6 × 10⁶ of cardiomyocytes were produced in a single run | Using MHC-neo ES cells | [31] |
|                      |            |              | Differentiation into neural lineage | 4.6 × 10⁶ of cardiomyocytes were produced in a single run | Using MHC-neo ES cells | [31] |
| 5.6 Stirred          | x          | x x x x x    | Differentiation into hepatic lineage | 4.6 × 10⁶ of cardiomyocytes were produced in a single run | Using MHC-neo ES cells | [31] |
|                      |            |              | Differentiation into osteogenic lineage | 4.6 × 10⁶ of cardiomyocytes were produced in a single run | Using MHC-neo ES cells | [31] |
| 5.7 Stirred +        | x          | x x x x x    | Differentiation into cardiac lineage | 4.6 × 10⁶ of cardiomyocytes were produced in a single run | Using MHC-neo ES cells | [31] |
| encapsulation (HA and |            |              | Differentiation into three germ layers | 4.6 × 10⁶ of cardiomyocytes were produced in a single run | Using MHC-neo ES cells | [31] |
| dextran) + perfusion | x          | x x x x x    | Differentiation into cardiac lineage | 4.6 × 10⁶ of cardiomyocytes were produced in a single run | Using MHC-neo ES cells | [31] |
| 5.8 Stirred +        | x          | x x x x x    | Differentiation into cardiac lineage | 4.6 × 10⁶ of cardiomyocytes were produced in a single run | Using MHC-neo ES cells | [31] |
| encapsulation (agarose) + perfusion | | | | | | |
| 5.9 Two type of        | x          | x x x x x    | Differentiation into cardiac lineage | 4.6 × 10⁶ of cardiomyocytes were produced in a single run | Using MHC-neo ES cells | [31] |
| stirred, STLV and static |            |              | Differentiation into cardiac lineage | 4.6 × 10⁶ of cardiomyocytes were produced in a single run | Using MHC-neo ES cells | [31] |
| suspension culture    |            |              | Differentiation into three germ layers | 4.6 × 10⁶ of cardiomyocytes were produced in a single run | Using MHC-neo ES cells | [31] |
| 5.10 RCCS (STLV and | x          | x x x x x    | Differentiation into cardiac lineage | 4.6 × 10⁶ of cardiomyocytes were produced in a single run | Using MHC-neo ES cells | [31] |
| HARV)                |            |              | Differentiation into cardiac lineage | 4.6 × 10⁶ of cardiomyocytes were produced in a single run | Using MHC-neo ES cells | [31] |
| 5.11 STLV             | x          | x x x x x    | Differentiation into cardiac lineage | 4.6 × 10⁶ of cardiomyocytes were produced in a single run | Using MHC-neo ES cells | [31] |
| 5.12 HARV+           | x          | x x x x x    | Differentiation into osteogenic lineage | 4.6 × 10⁶ of cardiomyocytes were produced in a single run | Using MHC-neo ES cells | [31] |
| encapsulation (alginate) |            |              | Differentiation into osteogenic lineage | 4.6 × 10⁶ of cardiomyocytes were produced in a single run | Using MHC-neo ES cells | [31] |
gravity-induced aggregation of the cells. For this reason, this technique has been used to generate plentiful cell types such as neuronal cells, hematopoietic cells, cardiomyocytes, vascular cells and chondrocytes. The hanging drop method is tremendously useful for appraisal of molecular mechanisms occurring in early embryogenesis in any cell type. However, this technique is mainly used for research purposes and is not suitable for large scale EB production because of its laborious nature; a typical 100-mm Petri dish can contain no more than 100 drops and each drop usually creates only one EB. Further limitations of this method include major difficulties in exchanging or manipulating the small volume of medium (less than 50 µL which can evaporate easily) without disturbing the EBs. Usually the hanging drop method is composed of two steps; the aggregation of ES cells in drops and maturation of aggregates to EBs in suspension culture using low adherence bacterial Petri-dishes. Several elements of the method may be troublesome such as loss of EBs during picking up the formed EBs by pipette and attachment of premature EBs on Petri-dishes.

Static suspension culture
Static suspension culture (Figure 1B) is used to produce a large number of EBs by simply inoculating a suspension of ES cells onto a bacteriological grade Petri-dish, ultra-low adherence plate or a Petri-dish coated with cell adhesion inhibitor such as poly 2-hydroxyethyl methacrylate (poly 2-HEMA), allowing the cells to spontaneously aggregate into spheroids. Although simple, this method allows little control over the size and shape of EBs. The result is frequent agglomerations of EBs into large, irregular masses because of the probability that ES cells encounter each other accidentally. An additional limitation of this technique is that EBs may prematurely attach to the plate because of the surface chemistry of the culture vessel, leading to a greater heterogeneity and loss of EBs from the suspension culture. On another hand, this method is popular for some applications such as differentiation of ES cells into the neuronal lineage.

Encapsulation/entrapment
Encapsulation/entrapment of a single cell suspension or
small clusters of ES cells in hydrogels (Figure 1C and D, respectively), such as methylcellulose[26], fibrin[33], hyaluronic acid, dextran[34], alginate[35], or agarose[36] represents a transition between hanging drop and static suspension approaches by generating individually separated EBs in a semi-solid suspension media. Entrapment of ES cells in methylcellulose, a temperature sensitive hydrogel, improves the overall synchrony and reproducibility of EB differentiation as it produces EBs of clonal origin. However, the efficiency of EB formation from individual ES cells can be rather low. In addition, soluble factor treatments and retrieval of differentiated cells may be complicated by the presence of the hydrogel material[38]. Interestingly, this method showed the possibility of designing a single cell culture system that would mimic the early developmental milieu and allow ES cells to switch between differentiation states within the same culture setting. When human ES (hES) cells are encapsulated in a 3D hyaluronic acid hydrogel, the hES cells can be maintained in an undifferentiated state. On the other hand, when hES cells are encapsulated in a dextran hydrogel, the hES cells are induced to differentiate and form EBs. Different types of hydrogels, therefore, act as a unique microenvironment for maintaining ES cells in either undifferentiated or differentiating state[31].

**Multiwell and microfabrication**

As an alternative approach for EB formation and culture, multiwell (Figure 1E and F) and microfabrication technologies have also been developed recently. Round-bottomed 96-well plates coated with or without reagents[39], 2-methacryloyloxyethyl phosphorylcholine (MPC)[38-40], glycosaminoglycan (CS)[41] and poly 2-hydroxyethyl methacrylate (poly 2-HEMA), have been utilized to prevent cell adhesion to the plastic surfaces. This technique is among the tools for forming EBs with high uniformity similar to the hanging drop method as a defined number of ES cells is seeded in the separated wells. In contrast to the hanging drop method, this technique has no requirement to exchange or manipulate the medium (approximately 200 µL) and it is easier to observe directly the EB formation with a microscope during cultivation. Because of these advantages, this technique may be used instead of hanging drop method for laboratory research. The forced aggregation system, involving centrifugation of ES cells within round-bottomed (U-shaped)[41,42] and triangle-bottomed (V-shaped) 96-well plates[43], can induce aggregation more rapidly than hanging drops. This procedure improves the reproducibility of EB production. On the other hand, it still requires individual processing and manipulation of the resulting EBs due to the requirement of one more additional plating step. Microwells fabricated by lithographic methods yield EBs in an equivalent or at a much higher density than other methods and allow preparation of size-controlled EBs in a scalable manner for reproducible of EB formation[44]. Likewise, batches of EBs can be formed in microfluidic chambers and separated from the flowing culture medium by a semi-permeable membrane, allowing for temporal control of the molecular makeup of the medium. The cell patterning method is also useful for high-throughput screening assays, such as the exploration of biochemical agents to direct aggregate-induced differentiation into a specific lineage without plating EBs[45].

**Bioreactors**

Stem cell-based technologies and tissue engineering possibly permit a wide span of clinical and biotechnology applications in future. Nevertheless, realization of the potential of stem cells will require their large-scale generation in a robust system without any limitation[46]. This highlights the requirement for the in vitro expansion of stem cells used for therapy prior to their commitment into tissue-specific applications. The potential of bioreactors to address this is demonstrated by their capacity to support a robust and well defined scale-up platform for expansion of ES cells[47], EB formation[48,49] as well as differentiation[50]. The scaling up of the design, given mass transfer limitations, will depend on the type of bioreactor chosen[51]. The theory of selecting bioreactors for stem cell expansion and differentiation beyond bench scale is largely reliant on whether the cells are adherent, suspension grown as single cells or aggregates for EB formation[52]. Therefore, bioreactor culture systems must be designed according to the application. In addition, bioreactors have a significant advantage over static suspension culture which are as follows: (1) scale up of expansion and differentiation of ES cells; (2) no labor-intensive requirements; (3) no space requirement for available area of ES cell growth; and (4) the ability to monitor and control critical culture parameters (i.e. pH, dissolved oxygen, glucose consumption, and lactic acid production)[53]. At the present time, EB formation in hydrodynamic conditions has been achieved by using bioreactors. They comprise (1) spinner flasks; (2) RCCS; (3) rotary orbital culture; and (4) complex methods combining these techniques. All of these techniques generally improve ES cell aggregation and form EB faster and more homogeneously in size compared to typical static suspension cultures.

**Spinner flasks**: Spinner flasks (Figure 1G) have been pioneered, as promising in vitro systems for stem cell expansion, EB cultivation and differentiation of ES/iPS cells into specific cell types[54]. Spinner flasks provide attractive benefits due to their simple design, scalable configuration, the flexible culture of cells as aggregates on microcarriers[55] or scaffolds[56], and ease of continuous monitoring for tight regulation of the culture environment (e.g. O2 tension, pH, shear forces, medium exchange rate)[57]. The simpler process in spinner flasks equipped with paddle-impellers results in the formation of large ES cells agglomerates within a few days[58]. The scaling-up is generally straightforward because of improved mass transport achieved by stirring. However, the flow environment created by the impeller renders them inappropriate, due to the shear stress[59]. Numerous culture parameters for this system have been optimized, including the agitation rate, cell initial concentration, me-
Recently, complex methods combining the above mentioned techniques have been adopted for solving the problems of these methods and keeping cells floating continuously in the culture medium. For example, the agglomeration of cells was avoided by keeping EBs in Petri-dishes for several days before transferring them into a different kind of environment; (1) spinner flasks; (2) a rotation culture system of Petri-dishes which were rotated on a horizontal rotation device; (3) rotary suspension culture in dishes on an orbital rotary shaker; (4) direct seeding ES cells into a spinner flask equipped with a glass ball bulb-shaped impeller or (5) two litres Stirred Tank bioreactor (STR) equipped with a newly developed pitched-blade turbine impeller.

In other cases, the encapsulation of ES cells was combined with transferring them into a bioreactor. For example, encapsulation of ES cells in defined conditions (i.e. number of cells per EB and capsule size); alginate,[65] agarose,[66], poly (lactic-co-glycolic acid)/poly (L-lactic acid) microsphere,[67,68], hyaluronic acid[69] and Matrigel[70] was used to control agglomeration of cells. Then, after the initial period of EB formation, all encapsulated ES cells were transferred to a spinner flask. The encapsulation system allowed a 61-fold expansion in the number of cells, similar to the static control non-encapsulated cell culture. Moreover, combination of the encapsulation of ES cells within alginate hydrogel, with or without 70s bioglass, followed by culturing cells in an HARV bioreactor directly enhanced both osteogenic differentiation in a functional test and generation of functional 3D mineralized constructs for further application of bone tissue engineering transplantation.[70,71] Finally, mES cells expanded as aggregates on microcarriers in stirred vessels retained expression of stem cell markers and could form EBs. Perfusion combined with frequent feeding has been shown to increase the expansion of ES cells and their differentiation into specific lineages, without compromising their stem cell performance.[72] Additionally, the effect of a rotary bioreactor promoted neural differentiation of hES cells in perfused and dialyzed STLV. The mean time delay for growing to so-called “neural rosette” formations was significantly shortened under STLV conditions compared to conventional static suspension culture. Likewise a perfused STLV bioreactor can decrease the expression of markers of undifferentiated stage and increase the expression of markers of differentiation, especially towards neural lineage commitments[73].

Recently, researchers have sought to develop culture systems with integrated bioprocesses, controlling stem cell expansion and differentiation tightly in a fully controlled bioreactor environment. For example, ten fold increase in expansion of ES cells as well as consequent neural differentiation was reported while drastically reducing, by 30%, the time required for differentiation process[64]. Moreover, microcarrier spinner flasks have been used for the culture of mES and hES cell expansion and directed differentiation. Mouse ES cells were allowed to proliferate on microporous collagen-coated...
dextran beads (Cytodex 3), glass microcarriers, and macroporous gelatin-based beads (Cultispher S) in spinner flasks. Under different inoculated cell densities and microcarrier concentrations, mES cells on microcarriers showed increased yield of approximately 70-fold (8 d) to about 190-fold (15 d). These cultured cells also successfully expressed Oct4, Nanog, and SSEA-1, and when dissociated from the beads, they formed EBs yielding cells with differentiation markers such as Flk-1, CD34 and α-MHC (mesoderm), HNF-3b19 (endoderm), and b3-tubulin57 (ectoderm).

**Computer-controlled bioreactors**

As mentioned before, the main advantage of computer-controlled bioreactors is process development by allowing online monitoring and control of specific culture parameters (temperature, pH, PO₂, lactic acid production and glucose consumption), and ensuring a fully controlled environment for stem cell cultivation. Oxygen-controlled bioreactors have been used for culturing mES and hES cell-derived cardiomyocytes. These experiments also assessed the effect of oxygen tension on cardiac differentiation which is a main concern. Moreover, this system was recently applied to culturing cells not only for stem cell expansion but also for differentiation. Expansion of a variety of stem cell types in bioreactors under defined and controlled conditions remains to be addressed. Future challenges also include the combination of expansion and directed differentiation steps in an integrated bioprocess that will ultimately result in scale-up of well differentiated cells to clinically relevant numbers.

It is worth mentioning that although differentiating cells in bioreactors have numerous benefits, these cells have been assessed for functionality by transplantation, and did not always perform well. Ten and twenty days post-implantation ES cells derived chondrogenic and osteogenic bioreactor aggregates showed no obvious influence on the healing process. In these experiments, all of the bioreactor derived cells showed higher Oct4 expression in the aggregates, even after 30 d of induced differentiation in a medium without LIF. This emphasizes the importance of proper condition set-up and timing during cultivation of cells in bioreactors.

**EMBRYONIC STEM CELL DIFFERENTIATION TO CARDIOMYOCYTES USING BIOREACTOR**

Regenerative medicine based on cell transplantation therapies has attracted increasing attention as a potential alternative to organ transplantation. Pluripotent stem cells (ES/iPS cells), because of their pluripotency and unlimited self-renewal capacity are promising cell sources to provide sufficient number of cells for therapeutic applications. However, the expansion and differentiation of these cells is still limited as a result of their complexity and difficult manageability in scale-up production for industrial purposes. To solve these problems, bioreactor culture systems offer advantages of ready scalability and relative simplicity.

Recently, a single-step bioprocess for ES cell-derived cardiomyocyte production have been developed by combining methods to prevent ES cell aggregation (hydrogel encapsulation) and to purify for cardiomyocytes from the heterogeneous cell populations by using genetic selection (myosin heavy chain-neomycin resistance; MHC-neo), with medium perfusion in a controlled bioreactor environment. It has been shown that the cardiomyocyte yield per input ES cells achieved in encapsulated culture was much higher than without encapsulation (3.17 ± 0.90 vs. 0.16 ± 0.07). Furthermore, higher cardiomyocyte yield was achieved under hypoxic conditions (4% oxygen tension) versus normoxia conditions (20% oxygen tension), when cultured in the stirred culture system. In addition, a 2-L bioreactor process enabling the controlled generation of EBs, derived from MHC-neo ES cell line, has been adopted for enhancing yield of ES-derived cardiomyocyte production. The fill-and-draw feeding protocol was replaced in a 2-L bioreactor, which allowed constant medium supply and avoided daily fluctuations of medium components. An optimized protocol resulted in more than five times greater cardiomyocyte yield, whereas medium consumption was 40% less than that in the control system.

For the controlled large-scale generation for clinical and industrial applications in humans, the efficacy of the dynamic process (Erlenmeyer, STLV bioreactor, Glass Ball Impeller (GBI) spinner flask and Paddle-Impeller (PI) spinner flask) compared to static suspension culture in Petri-dishes by analyzing the quality of EB formation and subsequent differentiation into cardiomyocytes. The EB rearrangement in the static system and EB cultivation in the GBI spinner flask resulted in higher EB yield, a round homogenous shape, the fastest growth rate and high contracting EB percentages over all other systems. As noted above, cardiomyocytes derived from ES cells are anticipated to be valuable for cardiovascular drug testing and disease therapies. However, the overall efficiency and quantity of cardiomyocytes obtained by differentiation of ES cells is still low. Recently, to enable large-scale culture of ES-derived cells, we have tested a scalable bioprocess that allows direct EB formation in a well controlled STLV bioreactor system. Our laboratory has developed protocols of cardiomyocyte differentiation from mES cells by using STLV. We have optimized the initial ES cell seeding density into the bioreactor, the rotation speed and the day of transferring and plating of EBs on gelatin coated Petri-dishes. We have compared the quantity and quality of EB production, as well as the efficiency of cardiac differentiation of samples derived form STLV, static suspension culture and hanging drop method. We found that the optimized rotary suspension culture method can produce a highly uniform population of efficiently differentiating EBs in large quantities in a manner that can be easily implemented by basic research laboratories (Figure 2). Although EBs derived from STLV start rhythmically contracting later than static suspension...
culture and hanging drop method, they beat with nearly 100% efficacy (Figure 3). Furthermore, our results are similar to other reports of EBs formed in STLV which were more uniform in size, and contained mostly viable cells whilst lacking necrotic centers. Additionally, STLV-produced EBs differentiated into cardiomyocytes more efficiently than those from static suspension culture[62]. Hence, this method provides a technological platform for the controlled large-scale generation of ES-derived cells for clinical and industrial applications.

CONCLUSION

Bioprocessing and commercialization of ES or iPS cells and tissue engineering products in cell replacement therapy have the potential to facilitate and transform breakthroughs from the research bench to the patient bedside. This is expected to be a long process, however, as there are many key practical issues to be addressed before moving ahead from the laboratory-scale fundamental research level. Laboratory-scale suspension cultures in hanging drops or Petri-dishes are useful tools for process development and initial optimization, and encapsulation/entrapment of ES cells, multiwell and microfabrication methods can improve high-throughput EB production. However, these approaches are not suitable for further therapeutic application because of their labor intensive, time consuming nature, culture-to-culture variability and lack of monitoring. Bioreactor culture systems address many of these problems and offer several advantages over the conventional use of basic culture methods for expanding and differentiating ES cells into specific lineages, without compromising their stem cell performance. Future challenges in bioreactor development will include the design of advanced and sophisticated monitoring platforms that allow monitoring at the cellular level of parameters including temperature, pH and oxygen levels. With respect to ES or iPS cells, we envision a scenario, where a complete bioprocess would exist in the bioreactor for the expansion and subsequent differentiation of the ES or iPS cells to generate the specialized cell type of interest. For example, the current achievements with cardiomyocytes derived from ES cells would be developed into cardiovascular grafts tissue engineering, with an emphasis on its possible clinical use in cardiovascular surgery. The engineering of a human cardiac tissue patch would be used to illustrate the biological requirements and engineering approaches for human applications. For future therapeutic application, the specialized cells differentiated from ES or iPS cells could then be used for cell therapies or combined with scaffolds to produce tissue construct and transplants for patients.

REFERENCES

1. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature 1981; 292: 154-156
2. Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by
teratocarcinoma stem cells. Proc Natl Acad Sci USA 1981; 78: 7634-7638

3 Thomson JA, Kalishman J, Golos TG, Durning M, Harris CP, Hearn JP. Pluripotent cell lines derived from common marmoset (Callithrix jacchus) blastocystcs. Biol Reprod 1996; 55: 254-259

4 Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waksnit MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. Science 1998; 282: 1145-1147

5 Takahashi K, Tanabe K, Ohmuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007; 131: 861-872

6 Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006; 126: 663-676

7 Park IH, Zhao R, West JA, Yabuchi A, Huo H, Ince TA, Lerou PH, Lensch MW, Daley GQ. Reprogramming of human somatic cells to pluripotency with defined factors. Nature 2008; 451: 141-146

8 Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K, Bernstein BE, Jaenisch R. In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature 2007; 448: 318-324

9 Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. Nature 2007; 448: 313-317

10 Ulhoa-Montoya F, Verfaillie CM, Hu WS. Culture systems for pluripotent stem cells. J Biosci Bioeng 2005; 100: 12-27

11 Doetschman TC, Eistetter H, Katz M, Schmidt W, Kemler R. The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. J Embryol Exp Morphol 1985; 87: 27-45

12 Hüpfl G, Gassmann M, Desbailliet I. Differentiating embryonic stem cells into embryoid bodies. Methods Mol Biol 2004; 254: 79-98

13 Itskovitz-Eldor J, Schuldiner M, Karsenti D, Eden A, Yanuka O, Amit M, Soreq H, Benvenisty N. Differentiation of human embryonic stem cells into embryoid bodies comprising the three embryonic germ layers. Mol Biol 2000; 6: 88-95

14 Mohr JC, Zhang J, Azarin SM, Soerens AG, de Pablo JJ, Thomson JA, Lyons GE, Palecek SP, Kamp TJ. The microwell system for pluripotent stem cell derivation: formation of embryoid bodies. Tissue Eng Part A 2009; 15: 1017-1030

15 Messana JM, Hwang NS, Coburn J, Elisseef JH, Zhang Z. Size of the embryoid body influences chondrogenesis of mouse embryonic stem cells. J Tissue Eng Regen Med 2008; 2: 499-506

16 Keller GM. In vitro differentiation of embryonic stem cells. Curr Opin Cell Biol 1995; 7: 862-869

17 Kurosawa H. Methods for inducing embryoid body formation: in vitro differentiation system of embryonic stem cells. J Biosci Bioeng 2007; 103: 389-398

18 Dang SM, Zandstra PW. Scalable production of embryonic stem cell-derived cells. Methods Mol Biol 2005; 290: 353-364

19 Barron V, Lyons E, Stenson-Cox C, McHugh PE, Pandit A. Bioreactors for cardiovascular cell and tissue growth: a review. Ann Biomed Eng 2003; 31: 1017-1030

20 KeohE DE, Lock LT, Parikh A, Tzanakakis ES. Propagation of embryonic stem cells in stirred suspension without serum. Biotechnol Prog 2008; 24: 1342-1352

21 KeohE DE, Jing D, Lock LT, Tzanakakis EM. Scalable Stirred-suspension Bioreactor Culture of Human Pluripotent Stem Cells. Tissue Eng Part A 2009; 15: Epub ahead of print

22 Cameron CM, Hu WS, Kaufman DS. Improved development of human embryonic stem cell-derived embryoid bodies by stirred vessel cultivation. Biotechnol Bioeng 2006; 94: 938-948

23 Gerecht-Nir S, Cohen S, Itskovitz-Eldor J. Bioreactor cultivation enhances the efficiency of human embryoid body (hEB) formation and differentiation. Biotechnol Bioeng 2004; 86: 493-502

24 Koike M, Sakaki S, Amano Y, Kurosawa H. Characterization of embryoid bodies of mouse embryonic stem cells formed under various culture conditions and estimation of differentiation status of such bodies. J Biosci Bioeng 2007; 104: 294-299

25 He Z, Li JJ, Zhen CH, Feng LY, Ding XY. Effect of leukemia inhibitory factor on embryonic stem cell differentiation: implications for supporting neuronal differentiation. Acta Pharmacol Sin 2006; 27: 80-90

26 Dang SM, Kyba M, Perlinoir E, Daley GQ, Zandstra PW. Efficiency of embryoid body formation and hematopoietic development from embryonic stem cells in different culture systems. Biotechnol Bioeng 2007; 98: 442-453

27 Takahashi T, Lord B, Schulze PC, Fryer RM, Sarang SS, Cuillan SR, Lee RT. Ascorbic acid enhances differentiation of embryonic stem cells into cardiac myocytes. Circulation 2003; 107: 1912-1916

28 Evans AL, Bryant J, Skepper J, Smith SK, Charnock-Jones DS. Vascular development in embryoid bodies: quantification of transgenic intervention and antiangiogenic treatment. Angiogenesis 2007; 10: 217-226

29 Kramer J, Hegert C, Guan K, Wobus AM, Müller PK, Rohwedel J. Embryonic stem cell-derived chondrogenic differentiation in vitro: activation by BMP-2 and BMP-4. Mech Dev 2000; 92: 193-205

30 Choi D, Lee HJ, Jee S, Jin S, Koo SK, Paik SS, Jung SC, Hwang SY, Lee KS, Oh B. In vitro differentiation of mouse embryonic stem cells: enrichment of endodermial cells in the embryoid body. Stem Cells 2005; 23: 817-827

31 Nonaka Y, Yoshikawa M, Oji Y, Matsuda R, Nishimura F, Yamada S, Nakase H, Moriya K, Nishio F, Ishizaka S, Sakaki T. CoCl2 inhibits neural differentiation of retinoic acid-treated embryoid bodies. J Biosci Bioeng 2008; 106: 141-147

32 Suslov ON, Kukekov VG, Ignatova TN, Steinzler DA. Neural stem cell heterogeneity demonstrated by molecular phenotyping of clonal neural progenitors. Proc Natl Acad Sci USA 2002; 99: 14506-14511

33 Liu H, Collins SF, Suggs L. Three-dimensional culture for expansion and differentiation of mouse embryonic stem cells. Biomaterials 2006; 27: 6004-6014

34 Gerecht S, Burdick JA, Ferreira LS, Townsend SA, Langer R, Vunjak-Novakovic G. Hyaluronic acid hydrogel for controlled self-renewal and differentiation of human embryonic stem cells. Proc Natl Acad Sci USA 2007; 104: 11298-11303

35 Magyar JP, Nemir M, Ehler E, Suter N, Perriard JC, Eppenberger HM. Mass production of embryoid bodies in microbeads. Ann N Y Acad Sci 2001; 944: 135-143

36 Kurosawa H, Imamura T, Koike M, Sasaki K, Amano Y. A simple method for forming embryoid body from mouse embryonic stem cells. J Biosci Bioeng 2003; 96: 409-411

37 Ezekiel UR. Single embryoid body formation in a multi-well plate. Electron J Biotechnol 2007; 10: 328-335

38 Koike M, Kurosawa H, Amano Y. A Round-bottom 96-well Polystyrene Plate Coated with 2-methacryloyloxyethyl Phosphorylcholine as an Effective Tool for Embryoid Body Formation. Cytotechnology 2005; 47: 3-10

39 Komno T, Akita K, Kurita K, Ito Y. Formation of embryoid bodies by mouse embryonic stem cells on plastic surfaces. J Biosci Bioeng 2005; 100: 88-93

40 Koike M, Sakaki S, Amano Y, Kurosawa H. Characterization of
embryoid bodies of mouse embryonic stem cells formed under various culture conditions and estimation of differentiation status of such bodies. J Biosci Biotechnol 2007; 104: 294-299

41 Ng ES, Davis RP, Azzola L, Stanley EG, Elefanty AG. Forced aggregation of defined numbers of human embryonic stem cells into embryoid bodies fosters robust, reproducible hematopoietic differentiation. Blood 2005; 106: 1601-1603

42 Ungrin MD, Joshi C, Nica A, Bauwens C, Zandstra PW. Reproducible, ultra high-throughput formation of multicellular organization from single cell suspension-derived human embryonic stem cell aggregates. PLoS One 2008; 3: e1565

43 Burridge PW, Anderson D, Priddle H, Barbadiello Muñoz MD, Chamberlain S, Allegrocci C, Young LE, Denning C. Improved human embryonic stem cell embryoid body homogeneity and cardiomyocyte differentiation from a novel V-96 plate aggregation system highlights interline variability. Stem Cells 2007; 25: 929-938

44 Moeller HC, Mian MK, Shrivastava S, Chung BG, Khademhosseini A. A microwell array system for stem cell culture. Biomaterials 2008; 29: 752-763

45 Sasaki D, Shimitzu T, Masuda S, Kobayashi J, Ioga K, Tsuda Y, Yamashita JK, Yamato M, Okano T. Mass preparation of size-controlled mouse embryonic stem cell aggregates and induction of cardiac differentiation by cell patterning method. Biomaterials 2009; 30: 4384-4389

46 Placzek MR, Chung IM, Macedo HM, Ismaili M, Mortera Blanco T, Lim M, Cha JM, Fauzi I, Kang Y, Ye DC, Ma CY, Polak JM, Panoskaltis N, Mantalaris A. Stem cell bioprocessing: fundamentals and principles. J R Soc Interface 2009; 6: 209-232

47 Kravetz R, Taiani JT, Liu S, Meng G, Li X, Kallos MS, Rancourt D. Large-Scale Expansion of Pluripotent Human Embryonic Stem Cells in Stirred Suspension Bioreactors. Tissue Eng Part C Methods 2009; Epub ahead of print

48 zur Nieden NJ, Cormier JT, Rancourt DE, Kallos MS. Embryonic stem cells remain highly pluripotent following long term expansion as aggregates in suspension bioreactors. J Biotechnol 2007; 129: 421-432

49 Cormier JT, zur Nieden NJ, Rancourt DE, Kallos MS. Expansion of undifferentiated murine embryonic stem cells as aggregates in suspension culture bioreactors. Tissue Eng 2006; 12: 3233-3245

50 Lock LT, Tzanakakis ES. Expansion and differentiation of human embryonic stem cells to endoderm progeny in a microcarrier stirred-suspension culture. Tissue Eng Part A 2009; 15: 2051-2063

51 Pörtner R, Nagel-Heyer S, Goepfert C, Adamietz P, Meenen NM. Bioreactor design for tissue engineering. J Biosci Biotechnol 2005; 100: 235-245

52 King JA, Miller VM. Bioreactor development for stem cell expansion and controlled differentiation. Curr Opin Chem Biol 2007; 11: 394-398

53 Niebruegge S, Bauwens CL, Peerani R, Thavandiran N, Masse S, Sevaptisidis E, Nanthakumar K, Woodhouse K, Husain M, Kumacheva E, Zandstra PW. Generation of human embryonic stem cell-derived mesoderm and cardiac cells using size-specified aggregates in an oxygen-controlled bioreactor. Biotechnol Bioeng 2009; 102: 493-507

54 Serra M, Brito C, Costa EM, Sousa MF, Alves PM. Integrating human stem cell expansion and neuronal differentiation in bioreactors. BMC Biotechnol 2009; 9: 82

55 Abranches E, Bekman E, Henrique D, Cabral JM. Expansion of mouse embryonic stem cells on microcarriers. Biotechnol Bioeng 2007; 96: 1211-1221

56 Vinjan-Novakovic G, Radisic M. Cell seeding of polymer scaffolds. Methods Mol Biol 2004; 238: 131-146

57 Zandstra PW, Bauwens C, Yin T, Liu Q, Schiller H, Zweigerdt R, Pasumarthi KB, Field LJ. Scalable production of embryonic stem cell-derived cardiomyocytes. Tissue Eng 2003; 9: 767-778

58 Schroeder M, Niebruegge S, Werner A, Willbold E, Burg M, Ruediger M, Field LJ, Lehmann J, Zweigerdt R. Differentiation and lineage selection of mouse embryonic stem cells in a stirred bench scale bioreactor with automated process control. Biotechnol Bioeng 2005; 92: 920-933

59 Chisti Y. Hydrodynamic damage to animal cells. Crit Rev Biotechnol 2001; 21: 67-110

60 Fok EY, Zandstra PW. Shear-controlled single-step mouse embryonic stem cell expansion and embryoid body-based differentiation. Stem Cells 2005; 23: 1333-1342

61 Leiknes PI, Unsworth BR. Neuroectodermal cell culture: endocrine cells. In: Atala A, Lanza RP, editors. Methods of tissue engineering. London: Academic Press, 2002: 371-382

62 Lu S, Liu S, He W, Duan C, Li Y, Liu Z, Zhang Y, Hao T, Wang Y, Li D, Wang C, Gao S. Bioreactor cultivation enhances NTES formation and differentiation of NTES cells into cardiomyocytes. Cloning Stem Cells 2008; 10: 363-370

63 Gerlach JC, Hout M, Edsbagge J, Bjoergvin P, Lübberstedt M, Miki T, Stachelscheidt H, Schmeler E, Schatten G, Zeilinger K. Dynamic 3D culture promotes spontaneous embryonic stem cell differentiation in vitro. Tissue Eng Part C Methods 2009; Epub ahead of print

64 Carpenedo RL, Sargent CY, McDevitt TC. Rotary suspension culture enhances the efficiency, yield, and homogeneity of embryoid body differentiation. Stem Cells 2007; 25: 2224-2234

65 Yirme G, Amit M, Laevsky I, Olsenberg S, Itskovitz-Eldor J. Establishing a dynamic process for the formation, propagation, and differentiation of human embryoid bodies. Stem Cells Dev 2008; 17: 1227-1241

66 Dang SM, Gerecht-Nir S, Chen J, Itskovitz-Eldor J, Zandstra PW. Controlled, scalable embryonic stem cell differentiation culture. Stem Cells 2004; 22: 275-282

67 Fernandes AM, Fernandes TG, Diogo MM, da Silva CL, Henrique D, Cabral JM. Mouse embryonic stem cell expansion in a microcarrier-based stirred culture system. J Biotechnol 2007; 132: 227-236

68 Carpenedo RL, Bratt-Leal AM, Marklein RA, Seaman SA, Bowen NJ, McDonald JF, McDevitt TC. Homogeneous and organized differentiation within embryoid bodies induced by microsphere-mediated delivery of small molecules. Biomaterials 2009; 30: 2507-2515

69 Levenberg S, Huang NF, Lavik E, Rogers AB, Itskovitz-Eldor J, Langer R. Differentiation of human embryonic stem cells on three-dimensional polymer scaffolds. Proc Natl Acad Sci USA 2008; 103: 12741-12746

70 Hwang YS, Cho J, Tay F, Heng JY, Ho R, Kazarian SG, Williams DR, Bocaccini AR, Polak JM, Mantalaris A. The use of murine embryonic stem cells, alginate encapsulation, and rotary microgravity bioreactor in bone tissue engineering. Biomaterials 2009; 30: 499-507

71 Zhang J, Wang M, Cha JM, Mantalaris A. The incorporation of 70s bioactive glass to the osteogenic differentiation of murine embryonic stem cells in 3D bioreactors. J Tissue Eng Regen Med 2009; 3: 63-71

72 Bauwens C, Yin T, Dang S, Peerani R, Zandstra PW. Development of a perfusion fed bioreactor for embryonic stem cell-derived cardiomyocyte generation: oxygen-mediated enhancement of cardiomyocyte output. Biotechnol Bioeng 2005; 90: 452-461

73 Côme J, Nissan X, Aubry L, Tournois J, Girard M, Perrier AL, Peschanski M, Cailleret M. Improvement of culture conditions of human embryoid bodies using a controlled perfused and dialyzed bioreactor system. Tissue Eng Part C Methods 2008; 14: 289-298
Akasha AA, Sotiriadou I, Doss MX, Halbach M, Winkler J, Baunach JJ, Katsen-Globa A, Zimmermann H, Choo Y, Hescheler J, Sachinidis A. Entrapment of embryonic stem cells-derived cardiomyocytes in macroporous biodegradable microspheres: preparation and characterization. *Cell Physiol Biochem* 2008; 22: 665-672

Taiani J, Krawetz RJ, Nieden NZ, Wu YE, Kallos MS, Matyas JR, Rancourt DE. Reduced Differentiation Efficiency of Murine Embryonic Stem Cells in Stirred Suspension Bioreactors. *Stem Cells Dev* 2009; Epub ahead of print

Wu K, Liu YL, Cui B, Han Z. Application of stem cells for cardiovascular grafts tissue engineering. *Transpl Immunol* 2006; 16: 1-7

Zweigerdt R, Burg M, Willbold E, Abts H, Ruediger M. Generation of confluent cardiomyocyte monolayers derived from embryonic stem cells in suspension: a cell source for new therapies and screening strategies. *Cytotherapy* 2003; 5: 399-413

Mummery C, Ward D, van den Brink CE, Bird SD, Doeveders PA, Opthof T, Brutel de la Riviere A, Tertoolen L, van der Heyden M, Pera M. Cardiomyocyte differentiation of mouse and human embryonic stem cells. *J Anat* 2002; 200: 233-242

Fong WJ, Tan HL, Choo A, Oh SK. Perfusion cultures of human embryonic stem cells. *Bioprocess Biosyst Eng* 2005; 27: 381-387

Bratt-Leal AM, Carpenedo RL, McDevitt TC. Engineering the embryoid body microenvironment to direct embryonic stem cell differentiation. *Biotechnol Prog* 2009; 25: 43-51

Yin CH, Chen W, Hsiao CC, Kuo CY, Chen CL, Wu WT. Production of mouse embryoid bodies with hepatic differentiation potential by stirred tank bioreactor. *Biosci Biotechnol Biochem* 2007; 71: 728-734

S- Editor Li LF  L- Editor Hughes D  E- Editor Lin YP

---

Rungarunlert S et al. Embryoid body and bioreactors