Video Article

Alginate Microcapsule as a 3D Platform for Propagation and Differentiation of Human Embryonic Stem Cells (hESC) to Different Lineages

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Abstract

Human embryonic stem cells (hESC) are emerging as an attractive alternative source for cell replacement therapy since they can be expanded in culture indefinitely and differentiated to any cell types in the body. Various types of biomaterials have also been used in stem cell cultures to provide a microenvironment mimicking the stem cell niche[1-3]. The latter is important for promoting cell-to-cell interaction, cell proliferation, and differentiation into specific lineages as well as tissue organization by providing a three-dimensional (3D) environment[4] such as encapsulation. The principle of cell encapsulation involves entrapment of living cells within the confines of semi-permeable membranes in 3D cultures[2]. These membranes allow for the exchange of nutrients, oxygen and stimuli across the membranes, whereas antibodies and immune cells from the host that are larger than the capsule pore size are excluded[5]. Here, we present an approach to culture and differentiate hESC DA neurons in a 3D microenvironment using alginate microcapsules. We have modified the culture conditions[2] to enhance the viability of encapsulated hESC. We have previously shown that the addition of p160-Rho-associated coiled-coil kinase (ROCK) inhibitor, Y-27632 and human fetal fibroblast-conditioned serum replacement medium (hFF-CM) to the 3D platform significantly enhanced the viability of encapsulated hESC in which the cells expressed definitive endoderm marker genes[1]. We have now used this 3D platform for the propagation of hESC and efficient differentiation to DA neurons. Protein and gene expression analyses after the final stage of DA neuronal differentiation showed an increased expression of tyrosine hydroxylase (TH), a marker for DA neurons, >100 folds after 2 weeks. We hypothesized that our 3D platform using alginate microcapsules may be useful to study the proliferation and directed differentiation of hESC to various lineages. This 3D system also allows the separation of feeder cells from hESC during the process of differentiation and also has potential for immune-isolation during transplantation in the future.

Video Link

The video component of this article can be found at https://www.jove.com/video/3608/

Protocol

All of the procedures below are carried out using aseptic techniques inside a Class II Biosafety Cabinet. Reagents and equipments used are listed in the tables below.

1. Preparation of 1.1% Alginate (w/v)

1. Add 0.275 g of purified sodium alginate (high glucuronic acid content ≥60%, viscosity >200 mPa s, and endotoxin ≤100 EU/g) in a sterile 50 ml tube and add 25 ml sterile 0.1% gelatin solution prepared earlier (0.5g gelatin/500 ml milli-Q H_2O and dissolved by autoclaving).
2. Vortex the tube for approximately 30 seconds to partially dissolve the alginate powder then place the tube on an orbital mixer at 10 x g for overnight (room temperature).
3. Add 2.778 ml of 9% sterile NaCl (4.5 g of NaCl/50 ml milli-Q H_2O) to 25 ml of alginate solution. Vortex the tube for approximately 30 seconds followed by centrifugation at 95 x g for 5 min.
4. Store the alginate solution at 4 °C for short-term storage (1-2 months) or at -20 °C for long-term storage (about a year).

2. Preparation of $CaCl_2$ Precipitation Bath

1. Dissolve 14.7 g of $CaCl_2 \cdot 2H_2O$ and 2.38 g of HEPES in 1 litre of milli-Q H_2O.
2. Adjust the pH level to 7.4.
3. Sterilize the solution using a 0.22 µm filter.
4. Precipitation bath can be stored at room temperature (6-12 months).

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3. Preparation of Decapsulating Solution

1. In 500 ml of Dulbecco's Phosphate Buffered Saline (D-PBS), add 50 ml of 0.5M EDTA and 5 ml of 1M HEPES.
2. Sterilize using a 0.22 µm filter or autoclave at 121 °C for 20 min.
3. Store the decapsulating solution at room temperature (6-12 months).

4. Preparation of Serum Replacement (SR) Medium

1. Prior to encapsulation, prepare SR medium in advance as described in Table of specific reagents and equipment.

5. Preparation of ROCK Inhibitor (Y-27632)

1. Dilute Y-27632 powder in 0.1% human serum albumin (HSA) in D-PBS to make a 5 mM solution.

6. Preparation of hESC for Encapsulation

1. Pre-treat the cells with culturing media supplemented with 10 µM of ROCK inhibitor (RI) for two hours at 37 °C (protect from light).
2. After the RI treatment, wash cells with D-PBS twice and dislodge the cells from culture plates enzymatically with accutase for 10 min at 37 °C.
3. Gently scrape the cells with the pipette or cell scraper and collect in a 15 ml tube. Neutralize the accutase with SR medium in 1:1 ratio.
4. To prepare a single cell suspension, filter the neutralized cells using a 40 µm filter and collect in a fresh 50 ml centrifuge tube.
5. Calculate the total number of cells in the solution using a hemacytometer.
6. Centrifuge the cell suspension 95 x g for 5 min and carefully discard the supernatant afterwards. Wash the cells with pre-warmed 0.9% NaCl. Centrifuge 95 x g for 5 min and discard the supernatant.

7. Encapsulation of Cells

1. Prepare a 1 ml syringe attached to a soft plastic tubing of 14G x 2" I.V. catheter. This is used to aspirate the cell suspension to be loaded on to the syringe pump as shown in Figure 1.
2. Resuspend the cells with pre-warmed alginate solution at a density of 1.25 million cells/ml alginate. Gently mix with the syringe and avoid creating bubbles.
3. Set up the bead generator, syringe pump and the air flow meter as shown in Figure 1. More details of these devices are listed in Table of specific reagents and equipment.
4. With the cells aspirated into the syringe, discard the plastic tubing of I.V. catheter and attach the syringe to the encapsulation machine. Ensure that there is a 10 cm gap between the end of the encapsulation machine and the collection point as shown in Figure 1.
5. Encapsulate the cells by setting the syringe pump at 20 ml/hr, air flow rate at 8 L/min and a pressure of 100 kPa (the size of capsules can be changed by altering the air flow rate).
6. Collect the encapsulated cells into a petri dish (100 x 15 mm) filled with 20 ml of pre-warmed precipitation bath for 7 min to stabilize the capsules.
7. Transfer the encapsulated cells by gentle aspiration into 50 ml centrifuge tubes filled with 20 ml of 0.9% NaCl.
8. Allow the capsules to settle into the bottom of the tube then gently discard the supernatant. Repeat the washing process with 0.9% NaCl.
9. Resuspend the encapsulated cells in pre-warmed culturing medium supplemented with RI (10 µM), transfer into a culture flask and incubated at 37 °C/5% CO_2.

8. Differentiation of Encapsulated hESC to DA Neurons

The encapsulated hESC are treated with RI for 3 days prior to differentiation.

1. Seed the mouse stromal cell line, PA6 cells at a density of 1.0 x 10^4 per cm^2 in 0.1% gelatin-coated T75 flask and condition the PA6 cells in DA neural differentiation medium (Materials Table) 24 hours before the differentiation.
2. Transfer the capsules to a 50 ml centrifuge tube and allow them to settle into the bottom of the tube.
3. Discard the supernatant and resuspend the capsules in PA6 cell-conditioned DA neural differentiation medium.
4. Culture the encapsulated hESC with the PA6 cell monolayer (9 x 10^6 hESCs per 7.5 x 10^5 PA6 cells) for 28 days with a media change on day 4 and every other day thereafter (change only half of the medium each time).
5. After 3 weeks in culture with PA6 cells, supplement the DA neural differentiation medium with 100 ng/ml SHH and 100 ng/ml FGF8a for the remaining week.

9. Decapsulation of Encapsulated hESC

1. Aspirate the capsules into a 15 ml centrifuge tube and allow them to settle at the bottom of the tube. Then discard the supernatant carefully.
2. Wash the capsules with D-PBS twice. Let the capsules settle at the bottom of the tube then remove the supernatant.
3. Add 5 ml decapsulating solution to the capsules. Mix the suspension thoroughly via aspiration prior to incubation at room temperature for 4-5 min.
4. Centrifuge the decapsulated cells at 95 x g for 3 min and discard the supernatant.
5. Wash the cell pellet with D-PBS followed by centrifugation at 95 x g for 3 min. Repeat.
6. The decapsulated cells can be further cultured as a monolayer or used for downstream analysis.

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10. Representative Results

The diameter of alginate microcapsules is 400-500 µm. The number of cells within the capsule was estimated by calculating the total number of cells divided by total number of capsules per run. Therefore, approximately 5.0 x 10^4 cells per capsule was estimated. From this, we presume that the maximum number of cells the capsule can contain is approximately 1.0 x 10^5. The viability of encapsulated hESC is >80% (Figure 2) as determined by using using carboxyfluorescein diacetate succinimidly ester (CFDA)/propidium iodide (PI) assay. We have optimized the conditions of hESC encapsulation by decreasing the alginate concentration from 2.2% down to 1.1% and by changing the precipitation bath from barium chloride to calcium chloride. From these conditions, we showed that only the cells which were encapsulated with 1.1% calcium alginate could survive, proliferate and form EBs *in vitro*[1]. To further optimize the condition, the effects of culturing media and RI, Y-27632 were investigated. The data as presented in Figure 2 and 3 demonstrate that RI prevented dissociation-induced apoptosis and maintained cell viability and promoted cluster formation[1,6]. Furthermore, the viability of encapsulated hESC cultured in hFF-CM + RI was significantly higher than other groups without RI supplementation; however this was not significantly different to encapsulated hESC cultured in SR + RI. Similarly, cell proliferation using BrdU assay increased from 25% to 75% as single cells developed into clusters (Figure 3). Apoptosis assay by TUNEL revealed that the single cells in microcapsules cultured in SR medium were apoptotic (data not shown) whereas the retrieved clusters from the hFF-CM were mostly negative for TUNEL. To a certain extent, hFF-CM supplemented with bFGF also promoted the survival and proliferation of encapsulated hESCs in the absence of Y-27632. However, treatment with Y-27632 before (2 hours) and after encapsulation (for an additional 4 days) markedly enhanced viability, proliferation and cluster formation of encapsulated hESC in 1.1% calcium alginate microcapsules.

Previously we demonstrated that encapsulated hESC can be successfully differentiated to definitive endoderm[1]. Here, we examined the application of cell encapsulation as a 3D platform to differentiate encapsulated hESC into DA neurons. hESC, that formed embryoid bodies (EB) in capsules were direct differentiated and on decapsulation under the conditions described showed a progressive neuronal morphology (Figure 4) after 2-3 days of culture with more than 90% viability. Gene expression analysis showed a down-regulation of pluripotent marker, OCT4 while neuroprogenitor marker, PAX6 and DA neuronal marker, TH were up-regulated after 7 days of differentiation (Figure 5A). Immunofluorescent staining revealed that differentiated hESC were PAX6-positive (>80%) but OCT4-negative at day 7. Further differentiated hESC showed TH-positive (>90%) neurons after 21 days (Figure 5B). Western blot analysis also showed an up-regulation of TH expression from day 14 (Figure 5C) while PAX6 expression was down-regulated after day 21. In comparison, the cells cultured under two-dimensional (2D) environment under similar conditions (e.g. RI pre-treatment for 2 hours and RI post-treatment for 3 days prior to differentiation) maintained a high percentage of PAX6-positive cells (>80%) throughout the differentiation period and were not as efficient in differentiating to TH-positive cells (<60%) as in 3D environment provided by encapsulation (Figure 5A and C).

Discussion

Several studies using mouse embryonic stem cells and hESC have demonstrated the benefits of 3D culture system in biomaterials and tissue engineering[2,3]. We used calcium alginate microcapsules as a suitable 3D platform to study hESC propagation and differentiation in comparison to barium alginate since hESC showed significant higher viability when encapsulated in calcium alginate than barium alginate. This culture system also allows a high-density cell culture and exchange of nutrients and oxygen across the membrane[7]. Spontaneous differentiation within the capsules has been previously observed and it is assumed that as undifferentiated hESC continue to proliferate within the capsules, the cells would eventually breakout of the capsules and form teratoma[1]. Another clinical application of encapsulation is to provide immune protection of transplanted cells from the host recipient. It is anticipated that transplantation of hESC and their derivatives may lead to immunological rejection since the low level of expression of MHC class I antigens of undifferentiated hESC is increased after differentiation[8]. It has also been documented that transplantation usually utilizes higher concentrations of alginate in order to circumvent the immune rejection process[2,9]. Our study uses a lower concentration of alginate (1.1%) which is more suitable for the *in vitro* culture and differentiation of hESC. It is yet to be determined whether the lower concentration of alginate we have used would illicit a similar immune response as previously reports as well as maintaining cell viability should these encapsulated hESC be transplanted in an immunocompetent host.

Our optimized encapsulation protocol for encapsulating hESC produces capsules size of 400-500 µm diameter. Capsules which are smaller than 400 µm tend to have fewer cells while larger capsules (>500 µm) result in an overpopulation of cells. hESC encapsulation requires a single cell formation, which also promotes cell apoptosis[6]. We have shown here that encapsulated hESC can continue to survive, proliferate and form EB. This is enhanced by pre-treating the hESC with RI prior to encapsulation, resulting in >80% hESC being viable. Thus, we have established a model to culture hESC in 3D culture conditions and have extended these studies for directed differentiation into DA neurons. Although cell encapsulation technique has been widely well-known for cell culturing and endodermal differentiation, neural differentiation under these conditions has not been studied thoroughly[10,11]. We have shown here that there is an increased expression of PAX6 and TH using gene and protein expression analyses after 7 days in comparison to 2D differentiation system, suggesting that the 3D environment promotes better DA neuronal lineage from pluripotent state. However, further analyses such as dopamine secretion test and transplantation assay are required to fully characterize the differentiated cells. Generating robust functional DA neurons efficiently is an essential requirement if cell therapy for Parkinson's disease is to become a reality. Our 3D platform as proposed about co-culturing with DA neural inducing cells, PA6 cells and high-density cell culture system of DA neuronal differentiated hESC via encapsulation is an effort towards that direction.

Disclosures

We have nothing to disclose.

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