Tissue Engineering

Embryonic stem cell sphere: A controlled method for production of mouse embryonic stem cell aggregates for differentiation

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ABSTRACT: *Objectives: Embryonic stem cells (ESCs) are of significant interest as a renewable source of nonproliferating cells. Differentiation of ESCs is initiated by the formation of embryoid bodies (EBs). Standard methods of EB formation are limited in their production capacity, in any variations in EB size and formation of EBs through frequent passages. Here we have reported the utility of a microencapsulation technique for overcoming these limitations by mass production of mouse ESCs in alginate beads called ESC spheres.*
Methods: *The mouse ESCs were encapsulated in 1.2% alginate solution and cocultured on a feeder layer. The cells were evaluated by flow cytometry, in vitro differentiation, immunofluorescence, and reverse transcriptase polymerase chain reaction (RT-PCR).*
Results: *Analysis of encapsulated ESC spheres by flow cytometry showed similar percentages of Oct-4 and stage-specific embryonic antigen-1 (SSEA-1) expression in comparison with routine culture of ESCs. Moreover, the ESC spheres maintained a pluripotency potential which was comparable with ESCs cultured on feeder cells directly, as demonstrated by immunofluorescence and RT-PCR.*
Conclusions: *The results demonstrated that alginate encapsulation as a simple bioreactor, provides a scalable system for mass undifferentiated ESC sphere production with similar sizes and without the need for frequent passages for differentiation and clinical and pharmaceutical applications.*
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KEY WORDS: *Alginate, Bioreactor, Differentiation, Embryonic stem cells*

INTRODUCTION

Mouse embryonic stem cells (ESCs) are clonal cell lines derived from the preimplantation embryos (1). They are able to differentiate to virtually all cell and tissue types *in vivo* as well as *in vitro* (2). The basic strategy for *in vivo* differentiation of ESCs is the formation of multicellular aggregates called embryoid bodies (EBs). EBs can be generated by using the hanging drop method, by culturing ESC colonies detached from cultures grown without a feeder cell layer, or by the spontaneous aggregation of ESC in suspension culture. These methods have some limitations, including (i) limited production of EBs, (ii) delivery of EBs with a large va-

riation in size and differentiation state, and (iii) the need for frequent passages. Moreover, frequent passaging conditions are associated with a heightened risk of contamination and, most importantly, may lead to uncontrolled cell-differentiation responses due to variation in the cellular microenvironment (3, 4). To facilitate the investigation and exploitation of ESC-derived cells in research, a scaling up of cell production and optimization of culture conditions are necessary (5). Microencapsulation of animal cells into hydrogels has been used with a large number of different cell types (6-8). Hydrogels are cross-linked hydrophilic polymers that contain large amounts of water without dissolution. They should allow nutrients and metabolic wastes to diffuse

through them, so that the encapsulated cells remain viable and functional while also allowing the molecules produced by the encapsulated cells to diffuse out to the environment. The naturally derived polymers (macromolecules) most frequently used as hydrogel scaffolds in tissue engineering are alginate, hyaluronate, collagen, and their derivatives (9). Alginates are a family of linear natural copolymers of 1,4-linked β-D-mannuronic acid (M) and α-L-glucuronic acid (G) of varying compositional and sequential structures (10, 11). They have several unique properties for cell microencapsulation, including a relatively inert aqueous environment within the matrix, a mild room temperature encapsulation process free of organic solvents (12), a high gel porosity which allows for high diffusion rates of macromolecules, and the ability to control this porosity with simple coating procedures (13).

Immobilization of ESCs into alginate microbeads in an undifferentiating condition is assumed to give rise to ESC spheres, which offers significant advantages over the hanging drop method and the spontaneous aggregation of ESCs. The immobilization process can be optimized to (i) provide continuous mass production of undifferentiated ESC aggregates for differentiation and (ii) deliver ESC aggregates with a narrow distribution in size and differentiation state without the need for frequent passages.

Here we describe a large-scale production of ESC spheres by immobilization of ESCs into alginate microbeads in an undifferentiating condition for tissue-engineering approaches. Microencapsulated ESCs can grow as compact colonies within the beads with the ability to differentiate. Alginate microbeads might be simple bioreactors which can lead to mass production of ESC spheres without the need for frequent passages for differentiation.

MATERIALS AND METHODS

Culture of undifferentiated ESCs

The ESC line Royan B1 (14) derived from the C57BL/6 mouse strain was used throughout the present study. ES cells were kept in an undifferentiated, pluripotent state using a mitomycin C–inactivated feeder layer (M0503; Sigma) of primary cultures of mouse embryonic fibroblasts (MEFs). They were cultivated on gelatin-coated (0.1%, G2500; Sigma) plastic flasks (Falcon) in ESC medium containing of knockout Dulbecco's modified Eagle's medium (DMEM; 10829-018; Gibco) supplemented with 15% ESC-

qualified fetal bovine serum (FBS; 16141-079; Gibco), 0.1 mM β-mercaptoethanol (M7522; Sigma), 2 mM glutamine (15039-027; Gibco), 0.1 mM non-essential amino acids (M7145; Sigma), 0.1 mM penicillin-streptomycin (15070-063; Gibco), and 1,000 IU/mL leukemia inhibitory factor (LIF; Esgro, ESG1107; Chemicon). Cultures were grown in 5% CO_2, 95% humidity, and were routinely passaged every 2 days.

ESC microencapsulation

For encapsulation, ESCs were isolated from MEFs by treatment of the cells with trypsin-ethylenediaminetetraacetic acid (EDTA) solution (15305-014; GIBCO), and replated for 1.5 hours onto precoated gelatin plates (0.1% W/V) containing ESC medium. The supernatant was then collected and centrifuged (6 minutes, 1,200 rpm). The isolated cells were resuspended in 1.2% alginate solution (A7003; Sigma) at 4×10^6 cells/mL. Beads were formed by dispensing the alginate/cell suspension dropwise into a 102 mM $CaCl_2$ solution via a 22-gauge needle attached to a syringe. After 10 minutes, the newly formed beads (70 beads containing approximately 40,000 cells/bead) were washed once with a sterile 0.9% saline solution[AUTHORS: please advise: phosphate-buffered saline (PBS)?] followed by 2 washes with ESC medium and 10 beads were cultured in each well of 12-well plate (TPP, 92412) in ESC medium on the MEF feeder layer. The cultures were maintained for up to 20 days at 37°C in a humidified atmosphere of 5% CO_2, with daily changes of medium. On day 3 after suspension of ESCs in alginate, embryonic spherical structures (ESC spheres) were observed in each well. From day 3 up to 20 days, alginate beads were transferred daily onto new feeder layers, and the number of ESC spheres emerging from the alginate beads was counted. Further, ESC spheres were collected for differentiation and flow cytometry to clarify the status of ESC spheres.

Flow cytometry

Expression of Oct-4 and stage-specific embryonic antigen-1 (SSEA-1) was considered as the indicators of undifferentiated ESCs and analyzed by 2-color flow cytometry. All staining was performed in staining buffer consisting of phosphate-buffered saline (PBS) supplemented with 1% heat-inactivated FBS, 0.1% sodium azide, and 2 mM EDTA. After determining the cell viability by trypan blue exclusion, cells were washed 2 times in staining buffer and fixed in 2%

paraformaldehyde for 15 minutes and then permeabilized in 0.5% Triton X-100 for 5 minutes. Non–specific antibody binding was blocked for 15 minutes at 4°C with a combination of 10% heat-inactivated rat and goat serum (prepared in our laboratory) in staining buffer. In each sample, 1×10^5 to 5×10^5 cells were incubated with appropriate primary antibodies or appropriate isotype-matched controls (eBioscience or Dako Cytomation) for 45 minutes at 4°C. Primary antibodies used here were anti-SSEA-1 (1:50, MAB4301; Chemicon) and Oct-4 (1:50, MAB1759; R&D Systems). The cells were then washed 2 times in staining buffer and incubated for 30 minutes at 4°C with fluorescein isothiocyanate (FITC)–conjugated goat F(ab')$_2$ anti-rat immunoglobulin (Ig) G2 (1:100, F6252; Sigma Immunochemical) and phycoerythrin-conjugated[AUTHORS: please advise: correct edit?] rat F(ab')$_2$ anti-mouse IgM (1:100, 12-5790; eBioscience) as appropriate. Flow cytometric analysis was performed with a BD-FACS Caliber Flow Cytometer (Becton Dickinson), and the experiments were replicated at least 3 times. Acquired data were then analyzed using WinMDI software.

Induction of differentiation

Bead-released ESC spheres at day 14-20, were first cultured suspended in ESC medium without LIF and feeder layer for 5 days and then plated on gelatin-coated dishes for 12 days in the same medium to form spontaneously differentiated cardiomyocytes. Formation of spontaneously differentiated neural cells was promoted by supplementation of the medium with retinoic acid (RA; R2625; Sigma) to a final concentration of 4 µM. In cases of routine culture of ESCs, differentiation was promoted by EB formation with hanging drops (800 cells/20 µL) and/or suspension culture of 5×10^4 cells/mL in bacterial dishes.

Fluorescent immunostaining

We used fluorescent immunostaining for evaluation of mouse ESCs[AUTHORS: please advise: correct edit?] and detection of their potency for differentiation. The cells were rinsed twice with PBS–0.05% Tween 20[AUTHORS: please advise: correct edit?] and fixed with 4% paraformaldehyde at 4°C for 20 minutes. The cells were permeabilized with 2% Triton X-100 in PBS, when required. The fixed cells were blocked for 1 hour at 37°C with 10% goat serum / PBS–0.05% Tween 20[AUTHORS: please advise: correct edit?]. Cells were incubated for 60 minutes at 37°C in a humidity chamber with primary antibodies against neuron-specific markers:[AUTHORS: please advise: correct edit?] β-tubulin-III (1:250, T8660; Sigma) and anti-MAP2 (M1406; Sigma), as well as the cardiomyocyte marker anti-α-actinin (A7811; Sigma). At the end of the incubation time, the cells were rinsed 3 times with PBS–0.05% Tween 20[AUTHORS: please advise: correct edit?] and incubated with the appropriate FITC-conjugated anti-mouse IgG (1:100, F9006; Sigma) secondary antibody for 60 minutes at 37°C. After rinsing with PBS, the nuclei were counterstained with propidium iodide (1 µg/mL, P4170; Sigma), and the cells were analyzed with a fluorescent microscope (BX51; Olympus, Japan).

Reverse transcriptase polymerase chain reaction analysis

Reverse transcriptase polymerase chain reaction (RT-PCR) was also performed to assess spontaneous differentiation of ESCs. Total RNA was collected from the cells using a Nucleospin RNA II kit (740955; Macherey-Nagel, Germany). Before reverse transcription, RNA samples were digested with DNAse I (EN0521; Fermentas) to remove contaminating genomic DNA. DNAse I was dissolved in $\times 10$ reaction buffer with $MgCl_2$, and 1 unit of DNAse I (1 U/µL) was added per 1 µg of RNA and incubated for 30 minutes at 37 °C. To stop DNAse I activity, 1 µL of 25 mM EDTA was added and incubated at 65 °C for 10 minutes. Standard reverse-transcription reactions were performed with 2 µg total RNA using oligo (dT)$_{18}$ as primer and a RevertAid H Minus First Strand cDNA Synthesis Kit (K1622; Fermentas) according to the manufacturer's instructions. Reaction mixtures for PCR, including 2.5 µL cDNA, $\times 1$ PCR buffer (AMS; Sinagen Company, Iran), 200 µM dNTPs, 0.5 µM of each primer pair, and 1 U *Taq* DNA polymerase (EP0403; Fermentas). The sequences of primers of β-tubulin, ESC marker (Oct-4), and cardiomyocytes (β-MHC, ANF, and MLC2V) have been published before (15), and the primers for neurons were Pax6, forward: 5'-GAGAGGACCCAT-TATCCAGATG-3', and reverse: 5'-GCTGACTGTT-CATGTGTGTTTG-3'; NKX2.2, forward: 5'-AGCCCTTTC-TACGACAGCAG-3', and reverse: 5'-GCGTCACCTCCA-TACCTTTCTC-3'; NKX6.1, forward: 5'-GACAAAGATGG-GAAGAGAAAAC-3', and reverse: 5'-GGTCCA-GAGGTTTGTTGTAATC-3'; β-tubulin III, forward: 5'-GTTCCACGTCTCCACTTCTTC-3', and reverse: 5'-CCAGGTCATTCATGTTGCTCTC-3'; Mash1, forward: 5'-GTCCTCTCCGGAACTGATG-3', and reverse: 5'-CAG-

GACGCCCGCCTGAAAG-3'. Polymerase chain reactions were performed in a Mastercycler gradient machine (Eppendorf, Germany). Amplification conditions were as follows: initial denaturation at 94°C for 5 minutes followed by 30 cycles (for β-tubulin 25 cycles) of denaturation at 94°C for 45 seconds, annealing for 45 seconds, extension at 72°C for 30 seconds, and a final polymerization at 72°C for 10 minutes. Products were separated on 1.5% agarose gel. The gels were stained with ethidium bromide (10 μg/mL) and photographed on a UV transilluminator (Uvitec, Cambridge, UK).

Statistic analysis

Flow cytometry data were compared using Mann-Whitney *U*-test[AUTHORS: please advise: correct edit?] with SPSS software. Results are expressed as the mean ± standard error of mean (SEM), and a p value ≤0.05 was considered to be statistically significant.

RESULTS

The hydrophilic nature of the alginate material enabled its rapid wetting by the culture medium, leading to efficient cell seeding, at a suitable cell concentration (4 million cells/mL), and homogenous distribution over the alginate pores (Fig. 1B). On day 3 after suspension of ESCs in alginate, the compact spherical aggregates formed could be called ESC spheres (Fig. 1C). The spheres began to emerge from day 3 (Fig. 1D, E), and the number of released ESC spheres progressively increased up to day 11 postencapsulation and
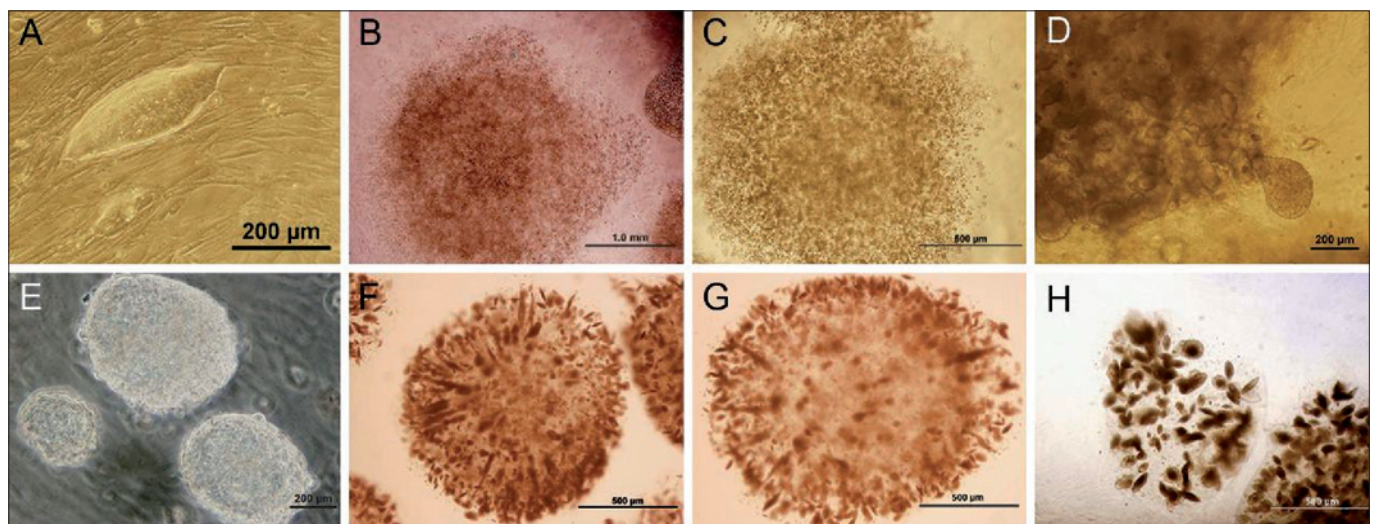

Fig. 1 - *Embryonic stem cell (ESC) proliferation in alginate beads. An ESC colony on a mouse embryonic fibroblast feeder layer (A). The hydrophilic nature of the alginate material enabled its rapid wetting by the culture medium, leading to efficient cell seeding at a suitable cell concentration, and homogenous distribution of the ESCs over the alginate pores. ESCs in alginate bead at day 1 (B) and day 3 (C) postencapsulation. On day 3 after suspension of ESCs in alginate, the compact spherical aggregates formed could be called ESC spheres which emerged out of the bead on day 3 (D) and released ESC spheres after that (E). ESC spheres in alginate beads at day 7 (F), day 14 (G), and day 20 (H) postencapsulation. The ESC sphere release gave rise to a reduction of the ESC pool in alginate beads.*

TABLE I - THE PERCENTAGE OF STEMNESS MARKERS IN ESC SPHERES DURING DIFFERENT DAYS

Markers	Day 3	Day 7	Day 10	Day 14	Control
Oct-4	99.81 ± 0.01	99.78 ± 0.02	99.79 ± 0.01	99.47 ± 0.24	99.83 ± 0.01
SSEA-1	99.62 ± 0.19	99.79 ± 0.04	99.80 ± 0.01	99.37 ± 0.26	99.82 ± 0.01
Oct-4 and SSEA-1 (double stain)	99.82 ± 0.01	99.69 ± 0.09	99.80 ± 0.02	98.84 ± 0.85	99.82 ± 0.01

Data are means ± SEM. There was no significant difference between different groups. For each sample, 1×10^5 to 5×10^5 cells were used. The experiments were replicated at least 3 times and analyzed 3 times. "Control" refers to the routine culture of ESCs on feeder cells directly in the presence of leukemia inhibitory factor, in comparison with encapsulated ESCs in alginate beads which were cocultured on feeder cells in the presence of leukemia inhibitory factor.
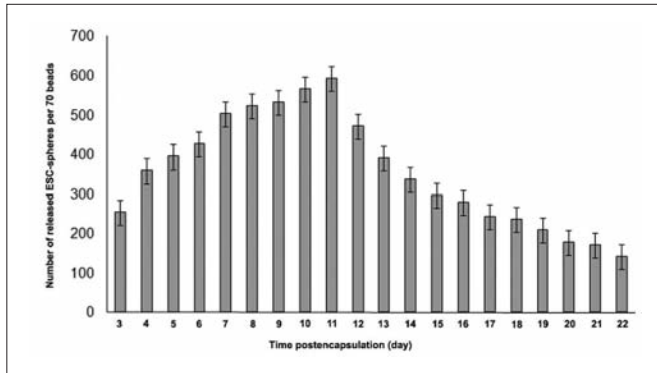SSEA-1 = stage-specific embryonic antigen-1.

decreased thereafter (Fig. 2).

To assess the percentage of cells with expression of stemness markers (Oct-4 and SSEA-1), flow cytometry analysis of ESC spheres at days 3, 7, 10, and 14 postencapsulation showed similar percentages of expression in both encapsulated and control groups (Tab. I; Fig. 3).

To evaluate the maintenance of the ESC spheres' pluripotency, their differentiation potential into cardiomyocytes and neural cells as ectodermal and mesodermal markers in ESC spheres and control ESC derivatives was assessed. Immunocytochemistry (Fig. 4) and RT-PCR (Fig. 5) analyses of differentiated cells showed the expression of cardiomyocyte markers including α-actinin (Fig. 4A), β-MHC, ANF, and MLC2V (Fig. 5), and neural cell markers: MAP-2 and β-tubulin III (Fig. 4B C) and Pax6, Nkx2.2, Nkx6.1, and Mash1 (Fig. 5).

DISCUSSION

ESC-derived cells have tremendous potential in many experimental and therapeutic applications. The ability to generate large numbers of the cells for differentiation in *in vitro* conditions is an important step toward clinical application. The most robust method for generating ESC-derived
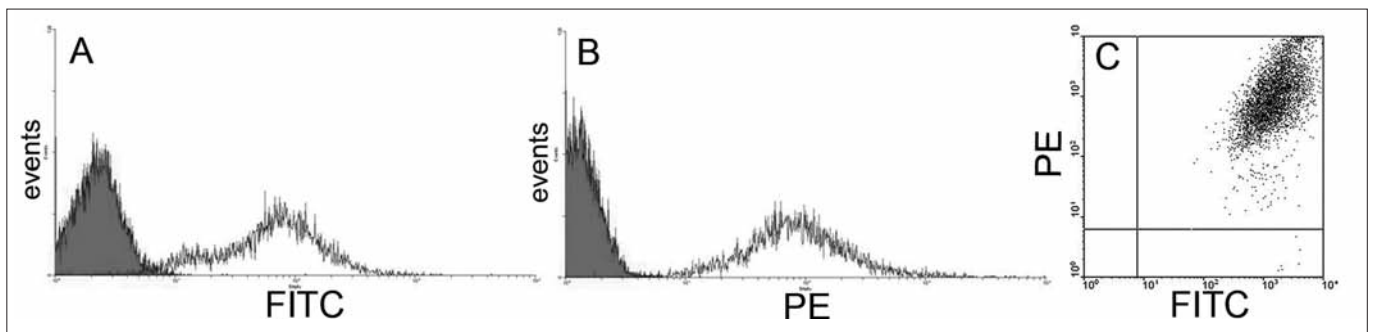

Fig. 3 - *Expression of Oct-4 and SSEA-1 in mouse ESCs maintained in alginate beads, as shown by flow cytometry. Representative samples of expression of Oct-4 (A), SSEA-1 (B), and both on ESCs (C). The cells expressing an antigen compared with isotype control (gray peaks) were termed positive populations. Percentages of double positives for Oct-4/SSEA-1 are indicated in the dot plots. Flow cytometry analysis of ESC spheres at days 3, 7, 10, and 14 postencapsulation showed similar percentages of expression in both encapsulated and control groups (Tab. I).*
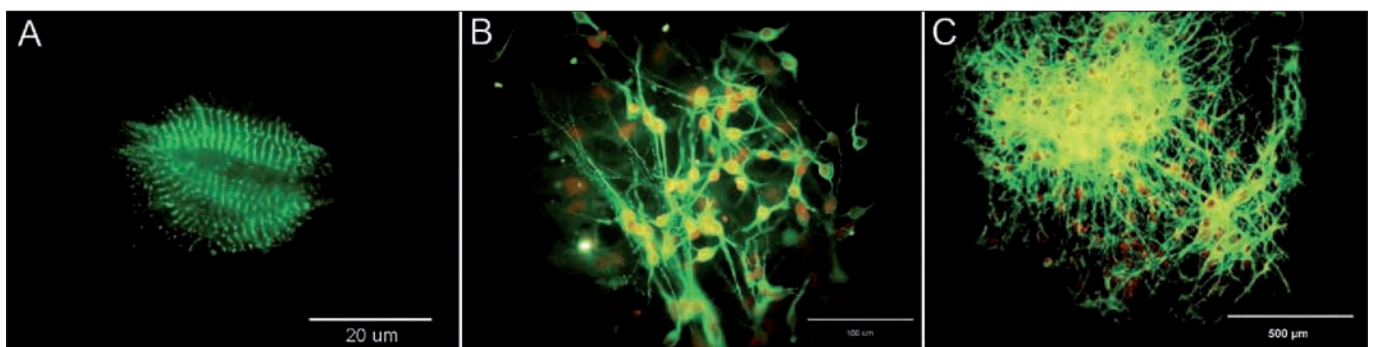

Fig. 4 - *Representative immunofluorescence of mouse ESC sphere–differentiated cells. Immunocytochemistry with anti-α-actinin (A), cardiomyocyte marker and anti-MAP2 (B), and anti-β-tubulin III (C) neuronal markers. This shows the maintenance of pluripotency in the ESC spheres.*
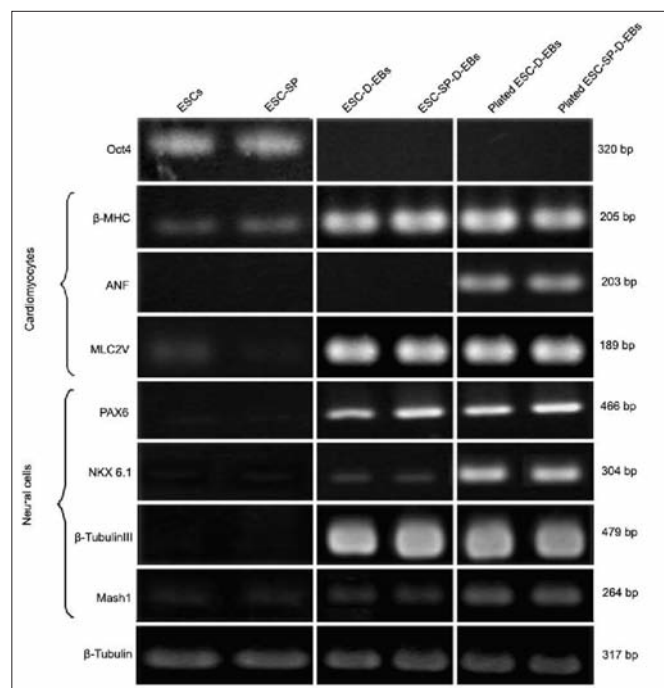
Fig. 5 - *RT-PCR analyses of differentiated cells derived from embryonic stem cell (ESC) spheres and/or controls which were cultured on mouse embryonic fibroblasts. Expression of cardiac-specific genes (β-MHC, ANF, and MLC2V) and neuronal-specific genes (Pax6, Nkx2.2, Nkx6.1, β-tubulin III, and Mash1) was observed as ectodermal and mesodermal markers in ESC spheres and control ESC derivatives. ESCs were co-cultured directly on feeder cells in the presence of leukemia inhibitory factor (LIF) or encapsulated in alginate beads and then cocultured on feeder cells in the presence of LIF to make ESC spheres (ESC-SPs). Then, ESCs or ESC-SPs were induced to differentiate by free-floating aggregates called embryoid bodies (EBs), by removing LIF and feeder cells, and to make ESC-derived EBs (ESC-D-EBs) and ESC sphere-derived EBs (ESC-SP-D-EBs). After 5 days the EBs were plated for another 12 days. Plated ESC-D-EBs = plated ESC-derived EBs; plated ESC-SP-D-EBs = plated ESC sphere-derived EBs.*

cells is through the EB system. Standard methods for EB formation (hanging drop and spontaneous aggregation of ESC in suspension) are limited in their production capacity and varying aggregate sizes. They also are not easily amenable to process-control strategies (5). The encapsulation of ESCs in hydrogel would overcome many of these current culture limitations. Encapsulation permitted the use of high cell density culture and production of ESC spheres through a controllable bioreactor system. In this study, we have tested the microencapsulation of ESCs into alginate microbeads. Our analysis of encapsulated ESC spheres which were cocultured on MEFs, by flow cytometry showed similar percentages of Oct-4 and SSEA-1 expression in comparison with routine culture of ESCs. Moreover, the ESC spheres maintained their pluripotency potential which was com-

parable with ESCs cultured on feeder cells directly, as demonstrated by immunofluorescence and RT-PCR. Therefore, alginate beads could be used as the scaffold bioreactor to generate scalable quantities of undifferentiated ESC aggregates with narrow distribution in size, and facilitate mass EB production (16). Indeed, our ESC spheres can be used as EBs in absence of LIF and feeder cells and can differentiate into embryonic germ derivatives. The scaffold provides a solid matrix along which the cells can adhere and interact with each other and with the solid matrix, while on the other hand, the medium-filled pores would allow the cells to aggregate in a manner similar to a suspension and/or a rotating cell culture system (17, 18). Applying a large-scale approach, it is important that the variability in the differentiation state of the EB is minimal. Using microencapsulation, 1 ESC is assumed to give rise to 1 ESC sphere that may be used instead of 1 EB. This offers significant advantages over the hanging drop, in which, either a defined number of cells or an unknown number of ESCs over a series of spontaneous aggregations are used for the formation of a single EB. Moreover, the monoclonality of bead-grown ESC spheres suggests the possibility to test large-scale gene-trap approaches in ESCs for identification of tissue-specific genes (19).

Recent investigations have reported a marked usage of alginate[AUTHORS: please advise: is this the correct phrase?] in the culture and differentiation of human ESCs, as well as in vasculogenesis (20) and in the formation of chondrocytes,[AUTHORS: please advise: correct edit?] cartilage tissue (21-24), mesenchymal stem cells (25-27), hepatocytes (28-30), parathyroid tissue (31), and islets of Langerhans (32, 33). It has therefore already been demonstrated previously that following alginate encapsulation, the cells retained their phenotype, increased the differentiation of stem cells, improved the function of cultured cells, and prevented cell-mediated rejection (32). Moreover, alginate is an interesting matrix for immobilizing and/or encapsulating proteins, with the main advantage being that the alginate gelation process occurs under very mild conditions[AUTHORS: please advise: correct edit?] without using high temperatures or chemical cross-linking agents (8). Alginate has been used to encapsulate angiogenic growth factors, such as basic fibroblast growth factor (bFGF) (34) and vascular endothelial growth factor (VEGF) (35-37) to treat ischemia. Alginate has also been found to have bioadhesive properties and can also be effective in protecting mucous membranes of the gastrointestinal tract (38). More recently, studies have described the generation

of scalable, controlled culture systems using alginate poly-L-lysine (PLL) encapsulation (39, 40).

In summary, we have demonstrated here that alginate encapsulation can be used as a simple bioreactor providing a scalable system for the mass production of ESC spheres with narrow distribution in their sizes, without the need for frequent passages for differentiation. This can therefore facilitate experimental designs requiring EB formation in a stem cell laboratory. The hydrophilic nature of the alginate scaffold, as well as its porous structure and interconnectivity, enables the ESCs to seed efficiently onto these scaffolds. In addition, unlike the routine method, this system may help to provide a renewable cell source needed for a variety of clinical and pharmaceutical applications without the need for frequent passages.

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