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Expansion and neural differentiation of embryonic stem cells in adherent and suspension cultures

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Abstract

The embryonic stem cell line, S25, is a genetically modified line that allows lineage selection of neural cells (M. Li, L. Lovell-Badge, A. Smith (1998) *Current Biology* **8**: 971–974). Here, the growth parameters of this cell line were analysed. Serial passaging in adherent conditions enabled these cells to grow rapidly (average specific growth rates of 0.035 h^{-1}) and generate high viable cell densities (above 90%). The aggregation of the S25 cells into embryoid bodies (EBs) was also studied, indicating limited cell growth (maximum cell densities of 2.7×10^5 cells ml^{-1}) and a high variability of aggregate size (70–400 μm after 8 d). Enzymatic dissociation of EBs with 1% (v/v) trypsin gave highest cell viability (91%) and density (1.4×10^4 cells ml^{-1}) and the cells thus obtained are able to differentiate into neurons.

Introduction

Embryonic stem (ES) cells are derived from the inner cell mass of the mouse epiblast and can contribute to all cell types of the embryo. They can differentiate *in vitro* into a wide variety of cell types including neurons, muscle and endothelial cells (Rathjen *et al.* 2002, Keller 1995). Furthermore, ES cells can generate all three neural lineages: neurons, astrocytes and oligodendrocytes (Bain *et al.* 1995, Fraichard *et al.* 1995), and the order of appearance of these cells during *in vitro* ES cell differentiation follows the same order in which they appear in the neuroectoderm of the embryo (Okabe *et al.* 1996, Li *et al.* 1998). These results suggest that neural precursors derived from ES cells can be used for experimental dissection of various aspects of mammalian neural development as well as a source for transplantation-based cell therapy.

Several methods have been described for the induction of neural differentiation in ES cells using stromal-derived factors (Kawasaki *et al.* 2000), conditioned medium (Rathjen *et al.* 2002) or retinoic acid (Bain *et al.* 1995). This later method is straightforward and involves the culture of ES cells in suspension conditions, forming characteristic floating aggregates – embryoid bodies (EBs) which, in presence of retinoic acid, leads to the conversion of undifferentiated ES cells to neural precursors. Although retinoic acid treatment results in a significative enrichment of neural cells in the differentiated progeny, several other cell types are produced. To circumvent this and obtain an almost pure population of neurons, Li *et al.* (1998) described a method that allows lineage selection of neural cells during ES cell differentiation. This method uses a genetically modified ES cell line, S25, where a selection marker, *β-geo* (a fusion between β-galactosidase and neomycin phosphotransferase) has been integrated into the *sox2* gene. Since this gene is active in neural precursor cells but not in differentiated cells, the culture of S25 ES cells in presence of geneticin results in the elimination of other differentiating cell types, and the isolation of a population

of morphologically immature cells that express neural markers (Sox1, Sox2, Nestin) (Li *et al.* 1998). In the presence of mitogens, these *sox2*-selected cells proliferate and retain their capacity for neuronal and glial differentiation, while in its absence, they differentiate into neurons and glia.

The use of genetically-modified ES cells to generate pure populations of neural cells and the ability to grow large numbers of these cells in culture would provide a safe, reproducible source of neural cells. These cells could then be used in animal models for testing neural drugs and in research and development toward cures for neurodegenerative diseases, such as multiple sclerosis, Huntington's and Parkinson's disease, which affect millions of people world wide and cost billions of dollars in health care each year.

In a first step to establish protocols for the large-scale expansion of S25 ES cells, we have analysed their growth conditions and the factors that influence the final number of neural cells that are generated. The key issues concerning the growth of mammalian cells are oxygen and nutrient transfer, growth conditions, and inoculation protocols. These factors, which influence the growth rate, expansion of stem cells as well as their differentiation potential, are addressed in this work.

Materials and methods

Cell line and media

The mouse embryonic stem (ES) cell line used in this work was the feeder-independent line S25 (derived from E14TG2a cell line), with a neomycin-resistance fusion introduced into the *sox2* locus by homologous recombination (Li *et al.* 1998, Billon *et al.* 2002), and was a gift from Meng Li and Austin Smith (Institute for Stem Cell Research, Edinburgh University, Scotland, UK). Cell culture was performed as described in Li *et al.* (1998) and Li (2002). ES cells were grown at 37 °C in a 5% (v/v) CO_2 incubator in Glasgow Modified Eagles Medium (GMEM), supplemented with 10% (v/v) foetal bovine serum, 2 ng leukaemia inhibitory factor ml^{-1} and 1 mM 2-mercaptoethanol.

To obtain EBs, S25 ES cells were plated in suspension conditions, using bacterial-grade petri dishes, in GMEM without leukaemia inhibitory factor (LIF). EBs were formed within 24 h and were treated with 1 mM all *trans* retinoic acid, at days 4 and 6 of culture, and geneticin (G418) from day 6 onwards. For differentiation into neural precursors, EBs were dissociated at day 8, and isolated cells were plated on poly-D-lysine and laminin coated dishes, in Dulbecco's modified eagles medium (DMEM) and nutrient mixture F-12 (Ham) in a 1:1 mixture (DMEM/F12) supplemented with N_2, 20 ng basic fibroblast growth factor ml^{-1}, 1 μg heparin ml^{-1}, 2% (v/v) chick embryo extract, and 200 μg geneticin ml^{-1}. For neuronal differentiation, EBs were dissociated at day 8, and isolated cells were plated on poly-D-lysine and laminin coated dishes in DMEM/F12 with N_2 supplement, without growth factors. After 1–2 d, the medium was replaced with Neurobasal medium supplemented with B27 (Gibco BRL).

Enzymatic dissociation procedures

Four protocols were compared for the dissociation of EBs, obtained after cultivating ES cells in suspension conditions for 4 d (initial cell density of 0.75×10^4 cells ml^{-1}). **Protocol 1** used an enzymatic dissociation technique with 1% (v/v) trypsin, as described by Li (2002). **Protocol 2** consisted in a mechanical dissociation, using a fine narrowed Pasteur pipette, as described by Reynolds & Weiss (1992). In **protocols 3** and **4**, EBs were enzimatically dissociated as described by Kallos *et al.* (1999), using either dispase (0.6 U ml^{-1} in PBS) or trypsin (0.05% v/v).

Clonal analysis

To determine if ES cells and cells derived from EBs exhibit self-maintenance, single ES cells and single cells resulting from EB dissociation were plated into agarose-coated individual wells of 96-well dishes and cultured for 8 d with GMEM (without LIF). Dishes were scored 24 h later. All wells containing one viable cell were marked and rescored 8 d later for the presence of aggregates.

Cell counts and viability

Cell density was determined using a hemacytometer. Viability was determined using the standard Trypan Blue dye exclusion test. All cell counts were performed in duplicate. Spherical aggregates were dissociated prior counting.

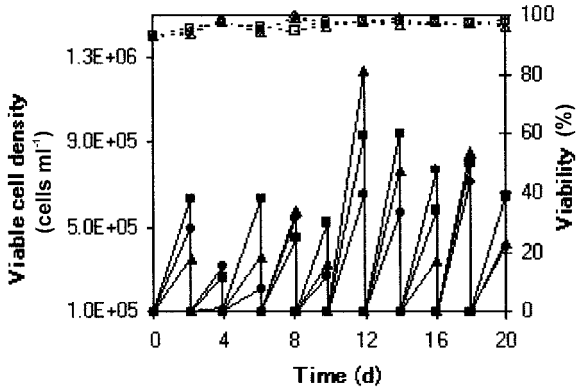

Fig. 1. The effect of serial passaging of S25 ES cells in stationary culture on viable cell density (■, ▲, ●) and viability (□, △, ○). The cells were grown in 35 (■, □), 60 (▲, △) and 90 (●, ○) mm dishes in GMEM supplemented with LIF. The cells were inoculated at 1×10^5 cells ml^{-1} and passaged every 2 d.

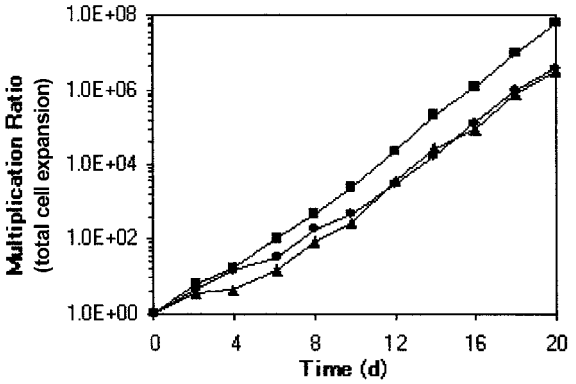

Fig. 2. Comparison of the multiplication ratios obtained with S25 ES cells in stationary cultures through serial passaging. The cells were grown in 35 (■), 60 (▲) and 90 (●) mm dishes in GMEM supplemented with LIF.

Aggregate size

Aggregate sizes of culture samples were determined by measuring, under an inverted microscope, of two perpendicular diameters for a minimum of 25 aggregates. The average diameter for each aggregate was calculated and then the mean for the sample was determined. Aggregates less than 20 μm in diameter (generally single cells or doublets) were not considered to aggregate statistical calculations.

Immunofluorescence

Cells were fixed in 4% (v/v) paraformaldehyde in PBS, and immunofluorescence was performed using standard techniques. Primary antibodies, anti *β-Gal* (ABCAM) and *Tuj1* (BabCO) were used at the dilutions of 1:1000 and 1:500, respectively. Secondary antibodies, Alexis-488 anti-rabbit and Alexis 594 anti-mouse, were used at the dilution of 1:400. Nuclei were labelled with 4′, 6-diamino-2-phenylindole dilactate. Cells were visualized in a Leica DMIRB fluorescence microscope and images were acquired with the Q-Fluor software (Leica).

Results and discussion

Effect of serial passaging

The main objective of this study was to examine the serial passaging of the genetically modified S25 ES cells and its effects on cell growth rates and viability. The scale-up of cell growth (using different size dishes, T-flask, spinner flask or bioreactors) involves serial passages, which could be potentially harmful to the cells. Figure 1 shows the results of serial passaging of ES cells in 35, 60 and 90 mm dishes, for 20 d. The volumes of media used were 3, 5 and 10 ml, respectively. The cells were always inoculated as an isolated cell suspension at 1×10^5 cells ml^{-1} and passaged every 2 d. The viability remained high (above 90%) during all the passages.

As a result of the passaging protocol, overall multiplication ratios, $MR = \Pi_{j=1}^{n} \frac{(\text{final viable cell density})_j}{(\text{initial viable cell density})_j}$ where j = passage number, of more than 10^6 were obtained over a 20 d period in adherent culture (Figure 2). Nevertheless, higher *MR* values were achieved for lower dish sizes.

The specific growth rate, $\mu = \frac{1}{n_t} \cdot \frac{dn_t}{dt}$ where n_t = total number of cells, doubling time $\left(D_t = \frac{\ln 2}{\mu}\right)$ for a first order kinetics, and maximum cell density are listed in Table 1. The calculated values were based on the initial and final cell densities. Within each experiment, only slight oscillations in the growth rate were registered. The comparison of the different systems indicates lower doubling times, higher growth rates and higher cell densities for 35 mm dishes, followed by the 60 mm dishes and then by the 90 mm dishes. These results are likely due to a decrease in the gas transfer efficiency concomitant with the increase in the dish size. In this type of system, the gas entrance occurs through the perimeter of the dishes. As the diameter of the dish increases, the ratio perimeter:area decreases, and, consequently, the gas entrance in the system be-

Table 1. Doubling times, specific growth rates and maximum cell densities obtained for the serial passaging of ES cells in stationary culture for 35, 60 and 90 mm dishes.

Passage number	Doubling time (h)			Specific growth rate (h^{-1})			Maximum cell density (10^5 cells ml^{-1})		
	35	*60*	*90*	*35*	*60*	*90*	*35*	*60*	*90*
1	18.4	26	20.8	0.038	0.027	0.033	6.33	3.52	4.89
2	31.1	231[a]	27.1	0.022	0.003[a]	0.026	2.63	1.2[a]	3.12
3	18.9	26.3	43.5	0.037	0.026	0.016	6.33	3.56	2.13
4	20.1	18.7	19.3	0.034	0.037	0.036	4.43	5.68	5.43
5	18.2	24.2	29.9	0.038	0.029	0.023	5.17	3.28	2.67
6	15.7	13.9	18.4	0.044	0.05	0.038	9.3	1.24	6.51
7	14.4	15.6	19	0.048	0.044	0.036	9.37	7.6	5.61
8	18.9	27.3	16.1	0.037	0.025	0.043	5.77	3.4	7.72
9	15.2	14.8	16.2	0.046	0.047	0.043	8.03	8.48	7.11
10	18	22.5	23.4	0.039	0.031	0.03	6.47	4.24	4.08
Average	18.9	21	23.4	0.038	0.035	0.032	6.38	5.8	4.93

[a]These values were not considered for the calculation of the averages.

comes lower, reflecting the importance of gas (O_2 and CO_2) mass transfer.

Similar results were obtained when the cells were seeded with the same number of cells per unit of area (data not shown). In this case, however, the multiplication ratio remained approximately constant for each dish size experiment. It was confirmed that the cells that were serially passaged continued to express the lineage marker (*β-Gal*) by immunofluorescence (data not shown). Together, these results show that S25 cells can be serially passaged, are able to grow rapidly and generate high viable cell densities.

Embryoid body formation

For embryoid bodies (EBs) to form, S25 ES cells were inoculated in bacterial grade petri dishes, so that attachment is inhibited and floating aggregates form. Within each EB, some of the cells start to differentiate spontaneously while others remain undifferentiated. The size of EBs varies greatly as a function of cell density and volume, sometimes reaching hundreds of microns in diameter. Very large aggregates are not optimal for cell growth and survival; therefore, aggregate size control is an important issue in the development of cell expansion protocols (Sen 2002).

The cell density and aggregate size of S25 derived EBs in stationary cultures were examined in 90 mm dishes, with two different volumes of medium (15 and 20 ml). Maximum cell densities of approx. 2.7×10^5 cells ml^{-1} being obtained in both cases. Samples of 1/10 of the total volume were collected and aggregate size measured. The results are shown in Figures 3 and 4. The size of EBs was highly variable, ranging from 70 μm to 400 μm, after 8 d in suspension culture. This could be responsible for the observed limited cell growth and survival: overall multiplication ratios (*MR*) of only approx. 4 were obtained over an 8 d period in suspension culture.

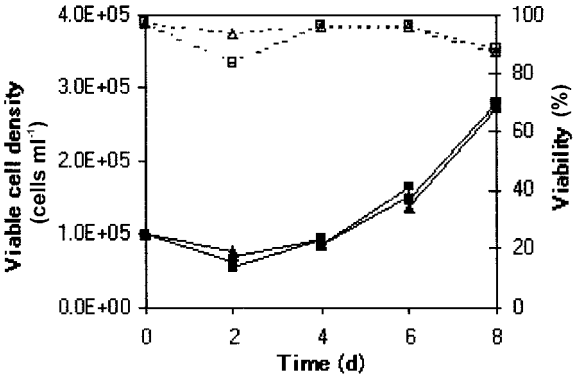

Fig. 3. Viable cell density (■, ▲) and viability (□, △) evolution of EBs grown in stationary culture. The cells were grown in 90 mm bacterial grade petri dishes, with 15 (■, □) and 20 (▲, △) ml of GMEM. Medium was changed and samples of 1/10 of the total volume were collected every other day.

These results show that S25 ES cells can be grown as aggregates in suspension culture, with high viabilities, although with low multiplication ratios. Better

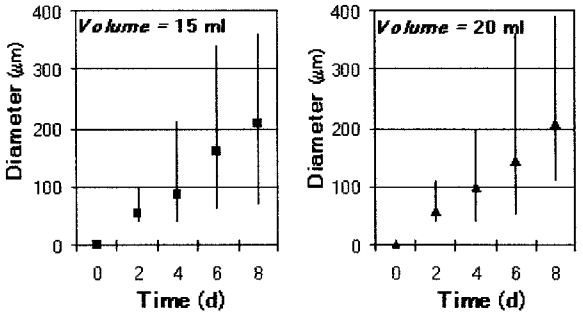

Fig. 4. Size evolution of EBs in stationary cultures. The cells were grown in 90 mm bacterial grade petri dishes, with 15 (■) and 20 (▲) ml of GMEM. Medium was changed and samples of 1/10 of the total volume were collected every other day.

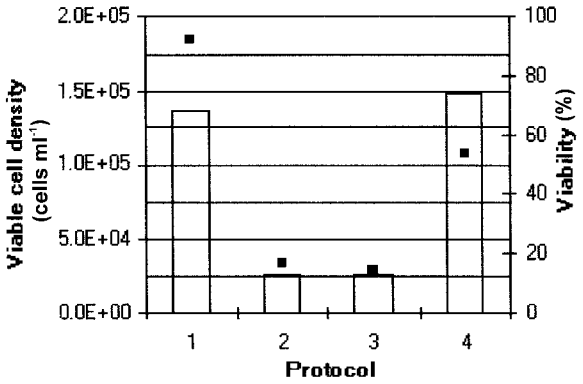

Fig. 5. Comparison of different protocols for the dissociation of EBs. The cells were grown in 60 mm bacterial grade petri dishes in GMEM, and dissociated as described in the text after 4 d. Bars represent the viable cell densities obtained and square symbols (■) represent the viabilities obtained.

results should be achieved using a non-stationary system, in which the agitation could be used to control the EB's diameter, and that would improve the gas and nutrients transfer through the aggregates.

Embryoid body dissociation

Several techniques for the dissociation of EBs have been reported (Reynolds & Weiss 1992, Kallos *et al.* 1999, Li 2002). In this study, enzymatic vs. mechanical methods of dissociating EBs were compared, in order to determine which was the most adequate for the cell line in use. The results are shown in Figure 5. After 4 d, the highest cell densities were obtained using trypsin (**protocol 1** and **4**). Within these two protocols, the highest viability (about 91%) was obtained using **protocol 1**. Trypsin is usually employed where integrity of cell surface proteins, like growth factor receptors, is important, which might be crucial for proper survival and differentiation of ES cells. On the contrary, dispase, an effective non-specific protease used for general cell dissociation, yields the lowest cell density and leads to the appearance of much cell debris. In our hands, mechanical dissociation also leads to poor survival and viability.

Overall, these results suggest that, at least for the S25 ES cell line, enzymatic dissociation with 1% (w/v) trypsin leads to the highest cell viability and density.

Embryoid bodies' potential

During S25 ES cell differentiation through EB formation, it is not known to what extent the cells within EBs retain the capacity to form new aggregates and also to differentiate into neural cells. To test the potentiality of S25 derived EB cells to form new aggregates, single EB dissociated cells were plated into agarose-coated individual wells of 96-well dishes, and cultured for 8 d in presence of GMEM (without LIF). This procedure resulted in the development of EBs in (49 $\pm$ 5)% of the wells that contained one viable cell at day 1. A similar result was obtained when single S25 ES cells were plated in the same conditions, leading to the formation of EBs in (51 $\pm$ 4)% of the wells. These results therefore suggest that S25 EBs can be amplified by dissociation of their cells and formation of secondary aggregates, allowing a greater number of precursor cells to be obtained.

To confirm that S25 derived EBs are able to give rise to neural precursors, dissociated cells were plated in a defined culture medium (DMEM/F12 with N_2 supplement) on poly-D-lysine and laminin coated dishes, in the presence of basic fibroblast growth factor, heparin, chick embryo extract and geneticin. Under these conditions, we observed that the majority of the cells continued to express the lineage marker (β-*Gal*) and that few cells exhibit markers of differentiating neurons, like Tuj1 (Figure 6A). On the contrary, when dissociated cells from S25 derived EBs were plated in conditions that promote neuronal differentiation (DMEM/F12 with N_2 supplement on poly-D-lysine and laminin coated dishes), most of the cells were Tuj1-positive, while few cells expressed the lineage marker β-*Gal* (Figure 6B). Thus, the S25 ES cells maintain their neural differentiation potential during EBs formation and cell growth in dissociation conditions.

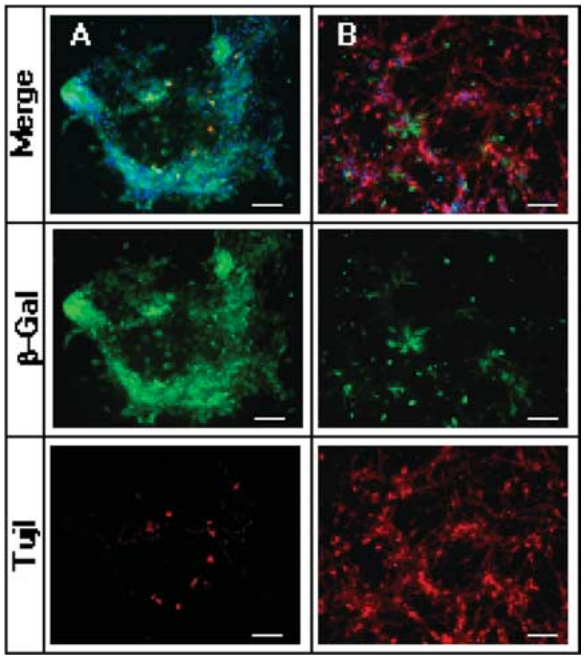

Fig. 6. Double-labelled immunocytochemistry with antibodies to *β-Gal* (green) and *Tuj1* (red), and nuclei labelled with 4′,6-diamino-2-phenylindole dilactate (blue): (A) plating of cells derived from EBs, on poly-D-lysine and laminin coated dishes in DMEM/F12 with N_2, in the presence of 20 ng basic fibroblast growth factor ml^{-1}, 1 μg heparin ml^{-1}, 2% (v/v) of chick embryo extract and 200 μg geneticin ml^{-1} (10 d); (B) plating of cells derived from EBs, on poly-D-lysine and laminin coated dishes in DMEM/F12 with N_2, followed by replacement with Neurobasal medium supplemented with B27 (9 d). Scale bar: 200 μm.

Conclusions

The work here described highlights some of the factors that might be crucial for the scale-up of S25 ES cell growth and their differentiation into neural precursors or neurons. A crucial aspect should be the control of EB formation which shows great variability in size and low cell multiplication ratios. The usage of a non-stationary system could probably lead to better results, as the diameter of the EBs could be controlled by the agitation rate and the diffusion gradients of nutrients and gas transfer could be minimized, thus improving cell growth within the aggregates. The capacity of S25 EB dissociated cells to form new aggregates suggests that it could be possible to amplify this population of precursor cells before neural differentiation. Finally, we confirmed that S25 ES cells can give rise to neural precursors or, alternatively, can be driven into neuronal differentiation with high efficiency. The capacity of S25 ES cells to generate both these cell types is essential when considering its use a potential source for transplantation-based cell therapy.

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