

## Optimization and integration of expansion and neural commitment of mouse embryonic stem cells

Maria Margarida Diogo\*, Domingos Henrique† and Joaquim M. S. Cabral\*<sup>1</sup>

\*IBB (Instituto de Biotecnologia e Bioengenharia), Centro de Engenharia Biológica e Química, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisbon, Portugal, and †IMM (Instituto de Medicina Molecular), Edifício Egas Moniz, Faculdade de Medicina da Universidade de Lisboa, Av. Prof. Egas Moniz, 1649-028 Lisbon, Portugal

To harness the potential of ES (embryonic stem) cells for human therapy, technology to develop the large-scale expansion and differentiation of these cells is required. In the present study, we tested various conditions for the expansion and neural commitment of mouse ES cells, using a cell line with a fluorescent reporter, which allows the monitoring of these processes by flow cytometry. The expansion of the 46C ES cell line in the presence of two different media [serum-free ESGRO Complete™ and DMEM (Dulbecco's modified Eagle's medium) containing 10% (v/v) fetal bovine serum] was compared. Both media yielded similar cell fold increases at two different initial cell densities and were able to maintain neural commitment potential during expansion. The influence of inocula concentration in the presence of two different media on cell proliferation and efficiency of neural commitment was evaluated. Two different chemically defined serum-free media were tested: the more conventional N2B27 and the second-generation medium RHB-A (Stem Cell Sciences, Edinburgh, Scotland, U.K.). The kinetics of neural commitment was followed during 8 days in the presence of both media. Our results show that inocula concentrations between  $5 \times 10^3$  and  $10^4$  cells/cm<sup>2</sup> are the most appropriate to achieve a better cell growth and more efficient neural commitment. We also show that cell culture in RHB-A medium results in higher rates of cell proliferation and neural commitment of ES cells, when compared with N2B27.

### Introduction

Mouse ES (embryonic stem) cells are continuous cell lines derived directly from the fetal founder tissue of the pre-implantation embryo [1]. One of the key properties of ES cells is their self-renewal capacity [1]. In fact, in the presence of the proper culture conditions, these cells can be expanded indefinitely as pluripotent populations, which maintain an undifferentiated phenotype. Another key property of ES cells is their pluripotency [1]. This means that these cells can be dif-

ferentiated *in vitro* into cells from the three embryonic germ layers that comprise an organism: mesoderm (muscle and bone), ectoderm (neurons and skin) and endoderm (liver and pancreas) through processes that recapitulate *in vivo* developmental programmes [1]. Furthermore, mouse ES cells are capable of re-entering embryogenesis when injected into a pre-implantation embryo, producing functional differentiated progeny in all tissues and organs [1]. Due to these properties, ES cell derivatives are potentially very attractive for many applications in cellular therapies, tissue engineering and drug screening. In particular, the generation of pure populations of neural progenitors from ES cells and their further differentiation into neurons, astrocytes and oligodendrocytes allows the potential use of these cells for the cure of neurodegenerative diseases and for drug testing. However, this type of application requires an available source of both undifferentiated ES cells and their neural differentiated derivatives, which constitutes an enormous challenge in terms of ES cell *in vitro* expansion and differentiation cultures. In fact, in order to develop large-scale cultures, the conditions for the *ex vivo* expansion and neural differentiation of ES cells have to be very well defined and optimized.

Stem cells can be defined as cells that must choose between alternative fates of self-renewal and differentiation at each division [2]. When the *in vitro* expansion of undifferentiated ES cells is intended, it is critical to maintain their pluripotency and self-renewal capacities. For this purpose, the derivation and expansion of mouse ES cells was originally carried out using a feeder layer of inactivated MEFs (mouse

Key words: cell expansion, cell proliferation, embryonic stem (ES) cell, neural commitment, serum-free medium.

Abbreviations used: ALP, alkaline phosphatase; BMP, bone morphogenetic protein; DMEM, Dulbecco's modified Eagle's medium; EBNA, transfected with Epstein-Barr nuclear antigen-1 gene; EBs, embryoid bodies; ES, embryonic stem; FBS, fetal bovine serum; FCS, fetal calf serum; GFP, green fluorescent protein; HEK-293, human embryonic kidney; hLIF, human leukaemia inhibitory factor; *Id*, inhibitors of differentiation genes; IRES, internal ribosome entry site; ; LIF, leukaemia inhibitory factor; Pen/Strep, penicillin/streptomycin; RA, retinoic acid; STAT3, signal transducer and activator of transcription 3.

<sup>1</sup> To whom correspondence should be addressed (email joaquim.cabral@ist.utl.pt).

embryonic fibroblasts) in the presence of serum-containing medium [3]. The function of this feeder layer is the provision of the LIF (leukaemia inhibitory factor) cytokine. LIF and related cytokines activate the latent transcription factor STAT3 (signal transducer and activator of transcription 3), which, in the presence of serum, is sufficient to direct ES cell self-renewal [4]. More recently, several feeder-independent mouse ES lines have been derived that can be maintained in the presence of a serum-containing medium supplemented with LIF [5]. Serum-containing medium, however, can cause cells to acquire karyotypic changes, is poorly defined and potentially exposes ES cells to animal pathogens. Several attempts have been made in order to develop serum-free formulations that are able to maintain ES cell properties during expansion. Previously, a specific complete serum-free medium was developed that maintains ES cell features during prolonged expansion, even when ES cells are expanded from single cells [2]. These characteristics include the potential to differentiate into cells of the three different embryonic germ layers, an unlimited and undifferentiated proliferative ability and a normal karyotype [2]. In the absence of serum, LIF is insufficient to block neural differentiation and maintain pluripotency. However, the authors found that, in addition to the LIF supplementation, the suppression of differentiation and concomitant efficient self-renewal requires only the additional molecular signals provided by BMPs (bone morphogenetic proteins) [2]. Indeed, these proteins were found to be responsible for the induction of *Id* (inhibitors of differentiation genes) that suppress neural determination [2]. Therefore *Id* contribute to self-renewal by complementing the blockade of other lineages by STAT3 [2].

To obtain a differentiated cell type from ES cells, it is first necessary to direct the commitment of these cells to a specific cell lineage and afterwards to develop conditions that support the viability and maturation of progenitor and terminal phenotypes. Great efforts have been made to develop methods for the efficient neural induction of mouse ES cells. The most widely used method is based on the formation of floating cell aggregates, called EBs (embryoid bodies), followed by treatment with RA (retinoic acid) in order to increase the frequency of neural derivatives [6,7]. It was previously described that ES cells differentiate as EBs to neuroectoderm in the presence of a chemically defined serum-free medium [8]. Although EBs recapitulate many aspects of cell differentiation during early embryogenesis, they are very disorganized and heterogeneous structures. Furthermore, RA should be avoided since it induces other lineages and affects positional specification [9]. Another system based on cell aggregation on suspension culture was also developed that allows the differentiation of ES cells to embryonic ectoderm in the presence of a conditioned medium called MEDII [10]. These cell aggregates are much

more organized when compared with EBs. However, MEDII constituents remain uncharacterized and the influence of cell aggregation on the process of differentiation is still very confused. Cell aggregation, however, is not necessary for ES cells to commit efficiently to a neural fate. In fact, neural cells can also be obtained on adherent conditions upon co-culture with a cell line of mouse stromal cells called PA6 [11].

All these culture systems that were described are still empirical and poorly reproducible since the mechanisms of neural commitment remain obscure and relatively inefficient. It is crucial to develop new systems in order to control efficiently the neural induction of ES cells. For this purpose, a very simple culture system based on the neural commitment of ES cells on adherent monolayer conditions was defined [9,12]. This very simple system allows direct observation of the process of neural conversion without the confounding influences of cell aggregation, co-culture, uncharacterized media constituents or cell selection [9]. The protocol is based on the adherent culture of ES cells in the presence of a serum-free chemically defined medium and in the absence of LIF after expanding the cells during 24 h at high density. In the absence of serum, withdrawal of LIF and other factors that promote ES-cell self-renewal (e.g. BMPs) results in the emergence of committed neuroepithelial cells. In order to examine and control the process by which ES cells acquire neural identity, a GFP (green fluorescent protein) knock-in reporter cell line (46C) was developed [12]. In this cell line, the open reading frame of the *Sox1* gene, an early marker of the neuroectoderm in the mouse embryo, was replaced with the coding sequence for GFP and an IRES (internal ribosome entry site)-linked puromycin resistance gene. The *Sox1* gene is first expressed in neural plate and neuroepithelial cells but is down-regulated during neuronal and glial differentiation. This double selection/resistance cassette allows both the monitoring of neural commitment (*Sox1*-GFP expression) by fluorescence microscopy and flow cytometry and the purification of neural precursors by puromycin treatment. This protocol of neural commitment was first optimized in the presence of a medium with a defined mixed formulation of basal media and supplements, the N2B27 medium, and using ES cells expanded in the presence of a serum-containing medium. The present study evaluates the influence of key factors on the expansion and neural commitment of mouse 46C ES cells. Factors such as the concentration of the inoculum, the constitution of the expansion and neural commitment media, the addition of a selection antibiotic and the integration of the expansion and neural commitment protocols were studied. Indeed, these are crucial parameters that have to be addressed in order to develop protocols for the large-scale expansion and neural commitment of mouse ES cells.

## Materials and methods

### Model ES cell line

46C mouse ES cells [12] were a gift from the laboratory of Professor Austin Smith at the Institute of Stem Cell Research at the University of Edinburgh (Edinburgh, Scotland, U.K.).

### Cell culture conditions

**Expansion of 46C mouse ES cells** Cells were cultured at 37°C, under 5% CO<sub>2</sub>, in a humidified incubator on 60 mm tissue-culture-grade dishes pretreated with 0.1% gelatin/PBS for a minimum of 30 min. Cells were plated in the presence of 5 ml of serum-free or serum-containing medium both supplemented (1:1000) with hLIF (human LIF) produced by HEK-293 (human embryonic kidney)-EBNA (transfected with Epstein-Barr nuclear antigen-1 gene) cells. HEK-293-EBNA cells were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) FCS (fetal calf serum), L-glutamine, Pen/Strep (penicillin/streptomycin) and 0.5 µg/ml puromycin. Cells were expanded in 20 cm × 15 cm TC plates and cultured for 48 h in DMEM supplemented with 10% (v/v) FCS, L-glutamine and Pen/Strep and for an additional 48 h in DMEM supplemented with L-glutamine and Pen/Strep. The supernatant was collected, centrifuged to remove the cell debris, filtered (0.2 µm) and aliquoted at -20°C. The formulation of the serum-containing medium is as follows: DMEM (Gibco) supplemented with 10% (v/v) FBS (fetal bovine serum; Gibco) ES cell qualified, 2 mM glutamine (Gibco), 1 × non-essential amino acids (Sigma), 1% Pen/Strep (Gibco) and 1 mM 2-mercaptoethanol (Sigma). The serum-free medium is the ESGRO Complete™ (Chemicon). Cells were cultured in the presence of these media and after 48 h they were dissociated using 0.025% trypsin solution (0.025% trypsin, 1.3 mM EDTA and 0.1% chicken serum, in PBS) at 37°C. The trypsin was neutralized with serum-containing medium, ES cells were spun down, resuspended directly in new medium and replated at the same density.

**Neural commitment of 46C ES cells** 46C ES cells were first expanded for two passages at  $1.2 \times 10^4$  cells/cm<sup>2</sup> and then for one passage at a relatively high density (10<sup>5</sup> cells/cm<sup>2</sup>) during 24 h in the presence of expansion medium (DMEM/FBS or ESGRO Complete™) supplemented with hLIF. After dissociation, the cells were resuspended in 1 ml of serum-free N2B27 or RHB-A (Stem Cell Sciences, Edinburgh, Scotland, U.K.) and replated on to 12-well tissue-culture-grade plates pretreated with 0.1% gelatin/PBS for a minimum of 1 h. The formulation of the N2B27 medium is as follows: DMEM/F12 (50:50) medium supplemented with modified N2 (25 mg/µl insulin, 100 µg/ml apotransferrin, 6 ng/ml progesterone, 16 µg/ml putrescine and 30 nM

sodium selenite) plus 50 µg/ml BSA fraction V, combined 1:1 with Neurobasal™ medium supplemented with B27 [9]. The RHB-A medium is a second-generation alternative medium to N2B27. The medium was changed every 2 days.

**Cell counts and cell viability** Cell counts were performed by using a haemocytometer and cell death was determined using the standard Trypan Blue dye exclusion test.

**Purification of Sox1-GFP-positive neural precursors by puromycin selection** The co-expression of Sox1-GFP and puromycin resistance gene allows the elimination of non-resistant cells in order to obtain 'clean' populations. After 4 days, puromycin (Sigma) was added *in situ* to the monolayer culture at a concentration of 0.5 µg/ml. This selection was maintained for 48 h.

**Monitoring of monolayer differentiation** The GFP fluorescence of Sox1-GFP<sup>+</sup> neural progenitors in serum-free medium was viewed using a standard fluorescence microscope. When the cultures were transferred to PBS for observation, the organizations typical of neural progenitors became more evident.

**FACS quantification of neural conversion** The use of 46C ES cells allows the quantification of neural progenitors by flow cytometry. The cells were firstly trypsinized, washed in serum-containing medium, spun down, resuspended in FACS buffer (PBS with 4% FBS) and counted. The cells were then analysed using the FACSCalibur™ flow cytometer (Becton Dickinson) and CellQuest software. Gates were set at 10 units of fluorescence, which excludes more than 99% of undifferentiated ES cells and dead cells. Cell debris and dead cells were excluded from the analysis based on electronic gates using forward scatter (size) and side scatter (cell complexity) criteria. All the settings were determined at the start of the experiment using undifferentiated 46C ES cells as negative control and ES cells expressing GFP as positive control.

**ALP (alkaline phosphatase) staining** 46C ES cells were washed with PBS and fixed in 10% unlabelled neutral-buffered formalin (Sigma) for 15 min. After fixing, the cells were washed and kept in distilled water for another 15 min. Following the washing step, the cells were incubated with a 1:1 solution of distilled water/Tris/HCl (Sigma) containing Naphthol AS MX-PO<sub>4</sub> (0.1 mg/ml; Sigma) and Fast Red Violet LB salt (0.6 mg/ml; Sigma) for 45 min and washed three times in distilled water. Finally, cells were kept in distilled water and observed with an optical microscope.

**Statistics and data analysis** The experimental results are presented as the means ± S.D. The non-paired Student's *t* test for independent samples was used to perform statistical analysis and a *P* value < 0.05 was considered significant.

## Results and discussion

### Comparison of the 46C ES cell growth in the presence of serum-free and serum-containing media

The first purpose of the present study was to evaluate the expansion of 46C ES cells in the presence of a serum-free medium and to compare cell growth in the presence and absence of serum. The influence of two different media (ESGRO Complete™ and DMEM containing 10% FBS) on the expansion of 46C ES cells was analysed. Cells were cultured in 60 mm tissue culture dishes at two very different initial cell densities:  $5 \times 10^4$  and  $4.3 \times 10^5$  cells/ml. These cell densities correspond respectively to  $1.2 \times 10^4$  and  $10^5$  cells/cm<sup>2</sup>. The lower initial cell density ( $5 \times 10^4$  cells/ml) was chosen taking into account the results previously obtained in our laboratory with a different ES cell line (S25) in the presence of a serum-containing medium when cells were expanded during 48 h. The higher initial cell density ( $10^5$  cells/cm<sup>2</sup>) was chosen because, according to the original protocol [9,12], ES cells should be plated at this density and expanded during 24 h prior to initiating the neural commitment. The average cell fold increases (ratio between the final and the initial number of viable cells) were calculated for duplicate determinations for four independent passages for each inoculum concentration. The cell viability was higher than 90% in all experiments (results not shown). With regard to the lower initial cell density ( $1.2 \times 10^4$  cells/cm<sup>2</sup>), after performing four passages during 48 h in each of the two different media, no statistically significant differences were found between the fold increases promoted by the two media. Indeed, average fold increases of  $10.8 \pm 1.9$  and  $11.5 \pm 2.9$  were obtained respectively for DMEM/FBS and ESGRO Complete™ media. The same conclusion was arrived at after performing four independent passages at high initial cell densities ( $10^5$  cells/cm<sup>2</sup>) during 24 h. In this case, average fold increases of  $2.3 \pm 0.8$  and  $2.3 \pm 0.7$  were obtained in the presence of DMEM/FBS and ESGRO Complete™ media respectively. This means that, for both culture conditions, the growth of 46C ES cells is very similar in either serum-free or serum-containing media. In the case of the inoculum of  $10^5$  cells/cm<sup>2</sup>, the average cell fold increases were 5–6 fold lower than that obtained for the inoculum of  $1.2 \times 10^4$  cells/cm<sup>2</sup>. This might be due to both the fact that cells were expanded only for 24 h and the very high initial cell density. This behaviour is similar to that described for the S25 cell line. In this case, the lower cell fold increase was also found for the higher tested inoculum concentrations ( $5 \times 10^5$  cells/ml or  $1.2 \times 10^5$  cells/cm<sup>2</sup>) due to surface area limitations. Indeed, in opposition to low inoculum cultures, high inoculum cultures were confluent at harvest.

These results show that, for two very distinct initial cell densities, it is possible to expand 46C ES cells in the

Table 1 Neural commitment efficiency of 46C ES cells after performing expansion in the absence and in the presence of serum

The percentages of Sox1–GFP<sup>+</sup> cells were obtained at day 6 of monolayer commitment of 46C ES cells in the presence of RHB-A or N2B27 medium when cells were expanded in the presence of DMEM/FBS or ESGRO Complete™ during two passages at  $1.2 \times 10^4$  cells/cm<sup>2</sup> during 48 h and one passage at  $10^5$  cells/cm<sup>2</sup> during 24 h. The cells were plated for neural commitment at an initial cell density of  $10^4$  cells/cm<sup>2</sup> and media were renewed every 2 days. Results shown are the means  $\pm$  S.D. for duplicate determinations from four independent experiments.

Expansion medium	Percentage of Sox1–GFP <sup>+</sup> cells in neural commitment medium	
	RHB-A	N2B27
DMEM/FBS	87.1 $\pm$ 6.0	85.6 $\pm$ 5.8
ESGRO complete™	91.9 $\pm$ 2.3	87.7 $\pm$ 5.3

absence of serum and that cell proliferation is very similar in the presence or absence of serum. Furthermore, future experiments on 46C ES cell expansion should be performed with an initial density of  $1.2 \times 10^4$  cells/cm<sup>2</sup> in the case of routine expansion studies since higher cell fold increase values are obtained. However, as recommended by the authors of [9,12], prior to initiating the neural commitment, 46C ES cells should be inoculated at a density of  $10^5$  cells/cm<sup>2</sup>.

### Comparison of the neural commitment potential of 46C ES cells expanded in the presence or absence of serum

In addition to cell growth, the two media (DMEM/FBS and ESGRO Complete™) were also compared in terms of their capacity to maintain the neural commitment potential during expansion. 46C ES cells were first expanded during three passages (two passages at  $1.2 \times 10^4$  cells/cm<sup>2</sup> and one passage at  $10^5$  cells/cm<sup>2</sup>) in the presence of ESGRO Complete™ or DMEM/FBS media. After expansion, cells were plated on to 12-well tissue culture plates at an initial cell density of  $10^4$  cells/cm<sup>2</sup> [9] in the presence of two different neural commitment media: RHB-A and N2B27. The neural commitment protocol was performed during 6 days, since previous results obtained with N2B27 [9] showed that the maximum percentage of Sox1–GFP<sup>+</sup> neural progenitor cells is obtained from day 4 until day 7. After 6 days of culture, the percentage of Sox1–GFP<sup>+</sup> cells was determined by flow cytometry. As shown in Table 1, no significant differences were found between the percentages of Sox1–GFP<sup>+</sup> cells obtained during the neural commitment protocol in the presence of RHB-A or N2B27 when cells were expanded in the presence of DMEM/FBS or ESGRO Complete™. Furthermore, a very high percentage of Sox1–GFP<sup>+</sup> cells (higher than 85%) was always obtained. Thus, it is possible to conclude that both serum-free and serum-containing media can be successfully used for 46C ES cells expansion while maintaining the cells' neural commitment potential.

After considering the results presented in the previous subsection of the 'Results and discussion' section and this subsection, and considering the advantages of using a serum-free medium for ES cell expansion, ESGRO Complete™ medium was selected for further expansion studies of 46C ES cells. The scalability of 46C ES cell expansion in the presence of this serum-free medium was already proved in our laboratory by using a spinner flask culture system with microcarriers [13]. Indeed, after expansion in this stirred system 46C ES cells retained their pluripotency and their neural commitment potential.

### Optimization of the inocula for neural commitment of 46C ES cells

As described in the previous subsection, the conversion of 46C ES cells into neural progenitors was performed using the adherent monolayer culture. In order to find the best initial cell concentration, cells expanded in the presence of the ESGRO Complete™ medium were plated at a density of  $5 \times 10^3$ ,  $10^4$ ,  $2 \times 10^4$  and  $4 \times 10^4$  cells/cm<sup>2</sup> on gelatin-coated 12-well plates in the presence of RHB-A and N2B27 media. The adherent monolayer culture was performed during 6 days. After 6 days of culture, total cells (Sox1–GFP<sup>+</sup> and Sox1–GFP<sup>-</sup> cells) were counted, total cell fold increase was calculated and the percentage of Sox1–GFP<sup>+</sup> cells was determined by flow cytometry. Averages of three independent experiments are shown for RHB-A and N2B27 media (Figure 1). As observed in Figure 1(A), in the presence of RHB-A, high percentages of Sox1–GFP<sup>+</sup> cells (> 87%) are obtained over a wide range of cell densities. However, according to statistical analysis, a significantly higher percentage of Sox1–GFP<sup>+</sup> cells was obtained for the inocula of  $10^4$  cells/cm<sup>2</sup> when compared with the other cell-plating densities. With regard to N2B27 (Figure 1B), no significant differences were found between the percentage of Sox1–GFP<sup>+</sup> cells obtained when cells were plated at  $5 \times 10^3$  or  $10^4$  cells/cm<sup>2</sup>. However, significantly lower percentages of neural progenitors were obtained for the inocula of  $2.0 \times 10^4$  and  $4.0 \times 10^4$  cells/cm<sup>2</sup> when compared with the two lower cell-plating densities. This lower efficiency of neural commitment observed for the higher cell-plating densities is probably due to the fact that high cell densities increase the potential for autocrine factors to maintain ES cells in an undifferentiated state [9]. By fluorescence microscopy, differences were also observed in terms of cell organization for the different cell-plating densities. For the lower cell densities ( $5 \times 10^3$  and  $10^4$  cells/cm<sup>2</sup>), clusters of ES cells differentiate into neural-tube-like structures (Figure 2A) and neural rosettes (Figure 2B) [9,12]. These types of organizations are typical of neuroepithelial cells, the source of all cells of the central nervous system. In opposition to this behaviour, for the higher inoculum concentrations ( $2 \times 10^4$  and  $4 \times 10^4$  cells/cm<sup>2</sup>), a lower number of these types of neuroepithelial

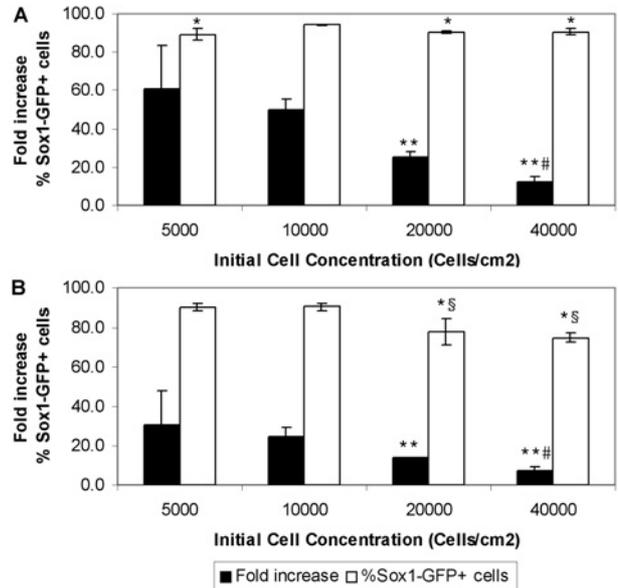


Figure 1 Neural commitment efficiency at different initial cell-plating densities

Effect of the initial cell density on the total cell fold increase (black bars) and percentage of neural commitment determined by flow cytometry (white bars) of 46C ES cells in the presence of RHB-A (A) and N2B27 (B). The cells were plated at initial cell densities of  $5 \times 10^3$ ,  $10^4$ ,  $2 \times 10^4$  and  $4 \times 10^4$  cells/cm<sup>2</sup> and cultured during 6 days. Media were renewed every 2 days. Results shown are the means  $\pm$  S.D. for three independent experiments. Statistically significant differences between the different initial cell-plating densities are denoted in the graph (\* $P < 0.05$  compared with  $10^4$  cells/cm<sup>2</sup> and § $P < 0.05$  compared with  $5 \times 10^3$  cells/cm<sup>2</sup> for the % Sox1–GFP<sup>+</sup> cells; \*\* $P < 0.05$  compared with  $10^4$  cells/cm<sup>2</sup>; and # $P < 0.05$  compared with  $2 \times 10^4$  cells/cm<sup>2</sup> for the total cell fold increase).

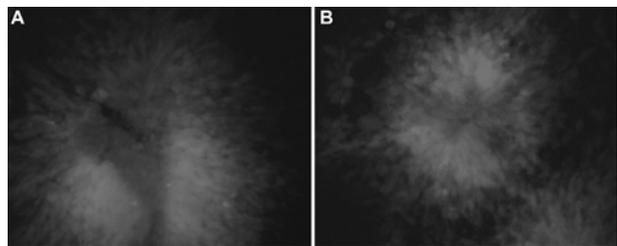


Figure 2 Expression of Sox1–GFP on neural-tube-like structures (A) and neural rosettes (B) of neural progenitors derived from 46C ES cells

This culture was performed during 5 days in the presence of RHB-A medium and transferred to PBS for observation.

structures can be observed. In these cases, in spite of the high percentage of Sox1–GFP<sup>+</sup> cells, most of the cells were visualized as a monolayer without any special type of organization.

When considering the cell-plating densities of  $10^4$ ,  $2 \times 10^4$  and  $4 \times 10^4$  cells/cm<sup>2</sup>, for both media, total cell fold increase was found to decrease when increasing inoculum concentration. This is an expected result since, as occurred

during cell expansion (subsection 'Comparison of the 46C ES cell growth in the presence of serum-free and serum-containing media'), higher initial cell densities are limited by surface area availability. With regard to the inoculum of  $5 \times 10^3$  cells/cm<sup>2</sup>, the averages of the total cell fold increase have high associated S.D. values, which probably means that small variations of the initial cell concentrations can cause dramatic changes of the total cell fold increase. Due to this, no significant differences were found when comparing this inoculum concentration with higher cell-plating densities. Taking into account the overall results, the inoculum concentration of  $10^4$  cells/cm<sup>2</sup> was selected for further experiments.

### Neural commitment kinetics of 46C ES cells

In order to compare the performance of N2B27 and RHB-A, the kinetics of neural commitment by cells expanded in the presence of the ESGRO Complete™ medium was followed during 8 days. Averages of three independent experiments are shown for RHB-A and N2B27 media (Figure 3). As observed by fluorescence microscopy, in the presence of both media, during the first 2 days, few cells become positive for Sox1–GFP. In fact, during this period, cells grow as clusters of undifferentiated ES cells. However, after 3–4 days, Sox1–GFP<sup>+</sup> cells are present. After 4 days of culture, most of the cells express Sox1–GFP and they assemble into the neuroepithelial structures described above in the subsection 'Optimization of the inocula for neural commitment of 46C ES cells' (Figure 2). The number of these cellular structures increases after 5 or 6 days of monolayer commitment. These observations on neural commitment were confirmed by flow cytometry analysis. Figure 4 shows an example of a flow cytometry profile and the respective quantification of the percentage of neural progenitors at day 6 of the monolayer commitment.

As observed in Figure 3(A), the maximum percentages of Sox1–GFP<sup>+</sup> cells are obtained after 6 days in the presence of both media and thereafter a progressive decrease in the percentage of Sox1–GFP<sup>+</sup> cells occurs until day 8. This slow decrease can be attributed to the differentiation of neural progenitors into neurons and glia [9, 12] that is accompanied by the down-regulation of Sox1–GFP. In fact, the Sox1–GFP<sup>+</sup> neural progenitors are a transient population during the differentiation of ES cells. When comparing the kinetics of neural commitment promoted by the two different media (Figure 3a), it was found that neural commitment begins earlier in the case of the RHB-A medium. Indeed, significantly higher percentages of neural progenitors were obtained from day 2 until day 4. The most significant differences are obtained on day 3, when the percentage of Sox1–GFP<sup>+</sup> cells is  $67.9 \pm 10.3\%$  in the presence of RHB-A and only  $35.8 \pm 5.7\%$  in the presence of N2B27. However, after day 6, similar high percentages of Sox1–GFP<sup>+</sup> cells are obtained for both

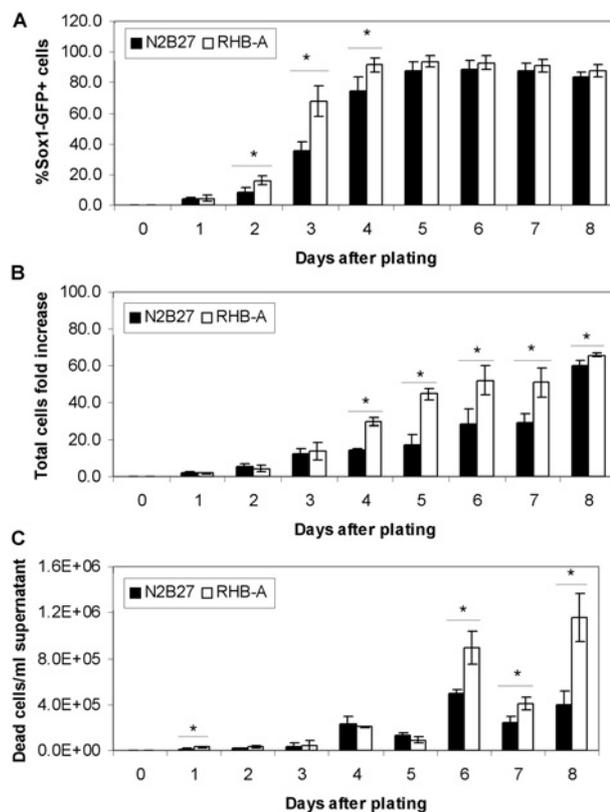


Figure 3 Kinetics of monolayer commitment of 46C ES cells

The percentage of Sox1–GFP<sup>+</sup> cells (A) and total cell fold increase for adherent viable cells (B) and the number of dead cells/ml in the supernatant (C) were determined for cultures performed in the presence of RHB-A (white bars) and N2B27 (black bars) media. The cells were plated at an initial cell density of  $10^4$  cells/cm<sup>2</sup> and the medium was renewed every 2 days. Results shown are the means  $\pm$  S.D. for three independent experiments. Statistically significant differences between RHB-A and N2B27 at specific time points are denoted in the graph (\* $P < 0.05$ ).

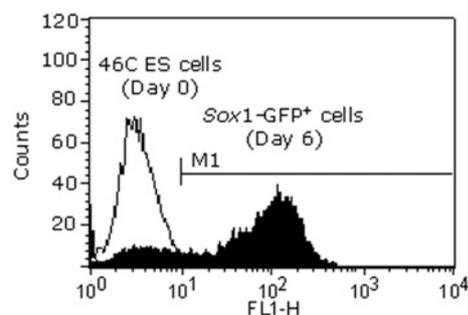


Figure 4 Flow cytometry profile of 46C ES cells at day 6 of the monolayer commitment and respective quantification of the percentage of Sox1–GFP<sup>+</sup> neural progenitors

This culture was performed in the presence of RHB-A medium. Cells were trypsinized, resuspended in FACS buffer (PBS with 4% FBS) and analysed using flow cytometry. All the settings were determined at the start of the experiment using undifferentiated 46C ES cells as the negative control and ES cells expressing GFP as the positive control.

media. Concerning the total cell numbers (Figure 3B), it is possible to observe that cell-plating efficiency was very high in the presence of both media. In fact, growth curves do not present lag phases and the initial cell viabilities are very high. This means that cells maintain a high viability when transferred to the serum-free neural commitment media. Furthermore, the viabilities of adherent cells determined during the 8 days of the experiment were always higher than 90% in the presence of both N2B27 or RHB-A. RHB-A and N2B27 media promote similar total cell fold increases until day 3. However, between day 4 and day 8, the RHB-A medium promotes significantly higher total cell fold increases. In order to compare cell death promoted by N2B27 and RHB-A, the number of dead cells was determined in the supernatant during the 8 days of the experiment by using the Trypan Blue dye exclusion test (Figure 3C). As observed in Figure 3(C), significantly higher numbers of dead cells were found for RHB-A on days 1, 6, 7 and 8. No significant differences were found on days 2, 3, 4 and 5. Thus it can be concluded that the higher number of total cells obtained in the presence of RHB-A from day 4 until day 8, when compared with N2B27, is due to a higher rate of cell proliferation promoted by RHB-A. Overall, these results show that the RHB-A medium is more suitable to perform the neural commitment of 46C ES cells.

### Purification of neural progenitors derived from 46C ES cells with puromycin

As mentioned above for the 46C ES cell line, the open reading frame of the Sox1 gene is replaced by a double selection/resistance cassette containing a puromycin resistance gene. Puromycin treatment was thus tested as a means of obtaining purer populations of neural progenitors. Since the levels of Sox1-GFP<sup>+</sup> cells reach their peak between day 4 and day 6 (Figure 3), puromycin treatment was included in the monolayer protocol during this period. In parallel, the monolayer protocol was also performed in the absence of puromycin. After 6 days, the percentage of neural progenitors and total cell fold increase was determined for both experimental conditions in the presence of RHB-A and N2B27. In the absence of puromycin, the percentages of Sox1-GFP<sup>+</sup> cells obtained were  $94.3 \pm 0.6\%$  for RHB-A and  $90.4 \pm 1.8\%$  for N2B27 (Figure 5). When puromycin was included in the culture medium, the percentages of neural progenitors obtained were  $96.9 \pm 0.5\%$  in the presence of RHB-A and  $89.9 \pm 0.8\%$  in the presence of N2B27 (Figure 5). After performing statistical analysis, a significant increase in the percentage of neural progenitors was found as a result of the puromycin treatment when monolayer was performed in the presence of RHB-A. In the presence of N2B27, however, puromycin treatment did not yield a statistically significant increase in the percentage of neural progenitors. Furthermore, no statistically significant

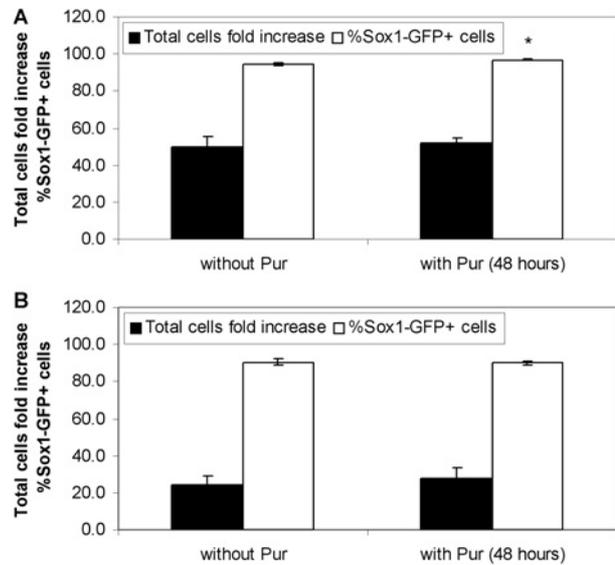


Figure 5 Purification of monolayer cultures with puromycin treatment

The total cell fold increase (black bars) and the percentages of Sox1-GFP<sup>+</sup> cells (white bars) were determined at day 6 in the presence of RHB-A (a) and N2B27 (b) media when the protocol of monolayer commitment was performed in the absence and in the presence (0.5 µg/ml) of a puromycin treatment from day 4 until day 6. The cells were plated at an initial cell density of  $10^4$  cells/cm<sup>2</sup> and medium was renewed every 2 days. Results shown are the means  $\pm$  S.D. for three independent experiments. Statistically significant differences between the absence and the presence of puromycin are denoted in the graph (\* $P < 0.05$  compared with absence of puromycin for the % Sox1-GFP<sup>+</sup> cells).

differences were found between the number of total cells obtained in the presence or in the absence of puromycin for RHB-A and N2B27. Thus the number of total cells was not reduced as a result of the puromycin treatment.

Although highly pure populations of neural progenitors can be obtained during monolayer culture, Sox1-GFP<sup>-</sup> cells still remain in the culture even after puromycin treatment. Since persisting undifferentiated ES cells are likely to be tumorigenic after transplantation, the presence of these cells was checked after performing the puromycin treatment during 48 h. For this purpose, total cells obtained after monolayer were replated at a density of  $10^5$  cells/cm<sup>2</sup> in the presence of a specific medium for the clonal-grade expansion of undifferentiated ES cells (ESGRO Complete™ medium). This medium had already been used for ES cells expansion described in the subsection 'Comparison of the 46C ES cell growth in the presence of serum-free and serum-containing media'. After 4 days, the presence of contaminating ES cell colonies was checked by performing an ALP (alkaline phosphatase) staining. ALP-positive colonies arose at a frequency of 0.03 and 0.6% when monolayer was performed in the presence of RHB-A and N2B27 respectively. In this way, it was concluded that puromycin treatment did not eliminate all undifferentiated ES cells.

However, once again, the RHB-A was proved to be superior to N2B27 for the neural induction of ES cells since it enabled us to obtain purer populations of neural progenitors much less contaminated with remaining undifferentiated ES cells.

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