Development of quantitative PCR methods to analyse neural progenitor cell culture state

Elsa Abranches*, Analeah O'Neill†, Matthew J. Robertson†, David V. Schaffer†[1] and Joaquim M. S. Cabral*

**Centro de Engenharia Biológica e Química, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal, and †Department of Chemical Engineering and the Helen Wills Neuroscience Institute, University of California, Berkeley, CA 94720-1462, U.S.A.*

Stem cells have significant potential for tissue engineering and regeneration, and neural stem and progenitor cells have proven promising for neuroregeneration in numerous animal disease and injury models. However, improved approaches must be developed to culture, expand and control the cells. Therefore the development of enhanced methods to quantify cell differentiation would significantly aid both in the basic investigation of cell-fate control mechanisms and in the optimization and validation of cell culture and expansion conditions. Quantitative reverse transcription–PCR methods were developed to quantify cell differentiation state by monitoring the expression of several cell-lineage-specific markers. These methods provide more rapid and readily quantitative results when compared with immunostaining. These methods were also applied in a preliminary investigation of cell-culture conditions, and it was found that regular feeding of cells with fresh medium is necessary to maintain them in an undifferentiated and highly proliferative state. The present study may aid both basic efforts to study the control of neural stem and progenitor differentiation as well as endeavours to optimize cell culture and expansion conditions for biomedical applications.

Introduction

Stem cells are a highly active area of exploration for numerous reasons. It is increasingly recognized that they play many crucial roles from development through adulthood, and gaining a deeper understanding of the mechanisms that control their behaviour can yield deeper basic biological insights. Furthermore, their hallmark ability to expand and differentiate into many specialized cell types makes them highly promising for tissue engineering and regeneration efforts. For both scientific endeavours and biomedical applications, the ability to analyse, validate and quantify the phenotypic state of cultured cells is fundamentally important.

Neural stem cells are capable of extended proliferation in the immature state and differentiation into the three major neural cell types of the nervous system: astrocytes, oligodendrocytes and neurons. In addition to a long-term, relatively quiescent neural stem cell, it is believed that populations of potentially shorter-term neural precursor exist throughout the nervous system. These multipotent cells, isolated from tissue and cultured into an actively dividing state, are sometimes referred to as neural progenitor cells. Neural stem and progenitor cells were first isolated from the embryonic CNS (central nervous system) [1] and peripheral nervous system [2]. However, it was subsequently shown that neural stem or progenitor cells actively divide and generate new neurons in several regions of the adult CNS [3–5] and that quiescent stem or progenitor cells potentially exist throughout it [6].

Neural stem cells have significant potential for replacing the neurons that progressively die in patients with chronic disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease, amylotrophic lateral sclerosis and multiple sclerosis, as well as acute neural trauma and stroke [7]. These cells can be expanded in culture for implantation at a site of injury. For example, cerebral implantation of expanded adult neural progenitor cells has resulted in significant reduction in demyelination and axonal loss in an animal model of multiple sclerosis [8]. Furthermore, implantation of an embryonic neural stem cell line into the site of spinal cord damage resulted in impressive functional recovery [9]. Alternatively, gene delivery to the CNS offers the possibility of modulating neural progenitor function in regions directly affected by neurodegenerative disorders, such as Alzheimer's disease [10].

Key words: cell expansion, differentiation, nestin, neural stem cell, progenitor, quantitative reverse transcription–PCR.

Abbreviations used: BrdU, bromodeoxyuridine; CNS, central nervous system; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FGF-2, fibroblast growth factor-2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; MBP, myelin basic protein; RT, reverse transcription; QPCR, quantitative PCR; QRT–PCR, quantitative RT–PCR.

[1] To whom correspondence should be addressed (email schaffer@cchem.berkeley.edu).

Clinical development of these and other applications will require robust methods to significantly scale-up immature progenitor cell cultures, and in some cases cells may need to be partially differentiated into a specific lineage prior to implantation [7]. A number of growth factors and hormones have been identified to regulate the proliferation [6,10–12] and differentiation [13–15] of neural progenitors in culture, and some culture condition optimization has been conducted for stem cells [16–18]. However, attempts to optimize conditions for the expansion and differentiation of stem cells would benefit from the development of robust, quantitative methods to evaluate the differentiation state of these cultures.

We have developed QRT–PCR [quantitative RT (reverse transcription)–PCR] methods to measure cell differentiation into the major neural lineages. Furthermore, to establish their utility, we have begun to apply these methods along with standard methods to quantify cell proliferation and viability to analyse the effects of culture conditions on cell culture state. We have found that regular feeding is necessary to prevent differentiation and maintain cells in an immature state. These methods and their validation are addressed in the present study.

Materials and methods

Cell culture

The cells used in the experiments were adult neural progenitors isolated from the hippocampus of adult female rats as previously described [6,10,15]. For cell-expansion studies, cells were grown in serum-free Ham's F-12/DMEM (Dulbecco's modified Eagle's medium) medium with N2 supplement (Invitrogen) and 20 ng/ml FGF-2 (fibroblast growth factor-2; Promega) on culture plates coated with polyornithine (Sigma) and mouse laminin (Invitrogen). For cell differentiation studies, cells were propagated in Ham's F-12/DMEM medium with N2 supplement, 0.5% (v/v) FBS (fetal bovine serum; BioWhitaker) and 0.5 μM retinoic acid (BioMol) on culture plates or multichamber slides (Fisher) coated with polyornithine and mouse laminin.

Cell counts and viability

For preliminary studies, cell density was determined using a haemacytometer. Viability was determined by using a standard Trypan Blue dye exclusion test. All cell counts were performed in triplicate.

These experiments revealed the need for more rapid measurements; therefore a method to quantify cell number and fraction undergoing DNA synthesis was pursued. Cells were first incubated for 16 h in 30 μM BrdU (bromodeoxyuridine; Sigma), a thymidine analogue incorporated into DNA during S-phase. Cells were then fixed, blocked with 4% (v/v) donkey serum (Sigma) in PBS, incubated with mouse anti-BrdU (1:100; Sigma) and stained with an Alexa-488-labelled secondary antibody (1:250; Molecular Probes). Finally, cells were incubated with propidium iodide (1:3000; Molecular Probes). Images of propidium iodide and BrdU staining were collected using a Leica fluorescence confocal microscope. Stained nuclei were automatically counted using Scion Image software, and results were checked by manual counting.

Immunofluorescence staining

Cells were fixed in 4% (w/v) paraformaldehyde in PBS, and staining was performed using standard techniques as previously reported [10]. The following primary antibodies were used: mouse anti-nestin (1:1000; Becton Dickinson), mouse anti-β-tubulin III (1:500; Sigma–Aldrich), mouse anti-Map2ab (1:500; Sigma–Aldrich), guinea-pig anti-GFAP (glial fibrillary acidic protein; 1:1000; Chemicon) and mouse anti-O4 (1:2; a gift from O. Boegler, Ludwig Institute for Cancer Research, London, U.K.). Detection of primary antibodies was performed with Alexa fluorochrome-conjugated secondary antibodies (1:250; Molecular Probes), and nuclei were stained with the molecular marker TO-PRO3 (Molecular Probes). Images were collected by fluorescence confocal microscopy (Leica Microsystems, Wetzlar, Germany).

QRT–PCR

Neural progenitors were cultured under proliferation or differentiation conditions, and their total RNA was subsequently isolated using the TRIzol® reagent (Sigma) and reverse transcribed using the ThermoScript™ RT–PCR system (Invitrogen). Addition of equal amount of RNA (1 μg) to each RT reaction was necessary to yield highly consistent results. We optimized QRT–PCR amplification and SYBR® Green DNA dye (Molecular Probes) detection conditions to quantify the transcripts of five genes known to be expressed at various stages in the process of neural precursor proliferation and differentiation. Initial PCR primer design was assisted using the program Vector NTI (Informatix) based on published sequence data (GenBank® database) of the following markers: GAPDH (glyceraldehyde-3-phosphate dehydrogenase; metabolic housekeeping gene), nestin (intermediate filament protein found in neural stem and precursor cell populations), β-tubulin III (cytoskeletal protein that serves as a neuronal marker), GFAP (intermediate filament protein expressed in astrocytes) and MBP (myelin basic protein; structural protein expressed in oligodendrocytes).

PCR reactions were carried out in a 20 μl volume containing 20 mM Tris/HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 μM of each primer, 1 μM fluorescein, SYBR® Green dye stock diluted down to 200

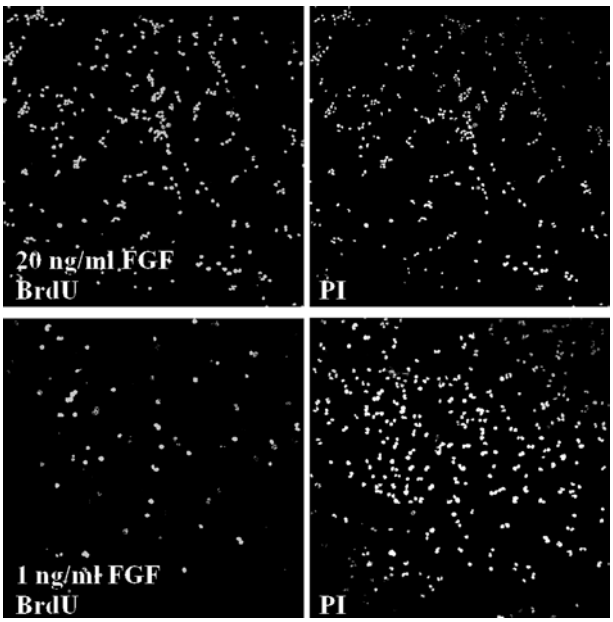

Figure 1 Images for quantification of cell proliferation by BrdU staining and total cell count by propidium iodide (PI) staining

The panels show the number of nuclei that have undergone S-phase DNA synthesis (i.e. BrdU incorporation), compared with the total number of nuclei indicated by propidium iodide staining. The top panels show a higher rate of DNA synthesis in 20 ng/ml FGF-2, as compared with 1 ng/ml FGF-2 in the lower panels.

times, 0.5 unit/μl Platinum Taq polymerase (Invitrogen) and 1 μl of template resulting from the reverse transcription reaction. About 40–50 cycles were performed in a Bio-Rad iCycler real-time PCR cycler, each consisting of 30 s denaturation at 94°C, 30 s primer annealing at 60°C (β-tubulin III), 65°C (GAPDH, GFAP and MBP) or 68°C (nestin), and 15 s elongation at 72°C. Results were analysed for statistical significance using a *t* test.

Results

Quantification of cell proliferation

The fraction of cells undergoing DNA synthesis was marked by BrdU uptake as an indicator of cell proliferation state. Labelled nuclei were immunofluorescently stained, and all nuclei were marked with propidium iodide. Fluorescence images were digitally collected and counted to determine total cells and the fraction of cells that had passed through the S-phase of the cell cycle during the 16 h BrdU pulse. For example, Figure 1 shows cells grown in 1 and 20 ng/ml FGF-2 resulting respectively in 17 and 95% of cells undergoing DNA synthesis during the BrdU-labelling period. Moreover, this standard method can be further adapted to measure cell viability.

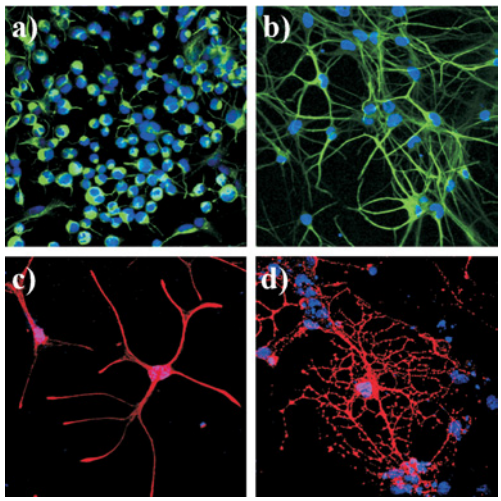

Figure 2 Immunostaining with antibodies against (a) the progenitor marker nestin (green), (b) neuronal marker β-tubulin III (green), (c) astrocytic marker GFAP (red) and (d) oligodendrocytes marker O4 (red)

Nuclei were labelled with TO-PRO3 (blue). Similar results were obtained with the neuronal marker Map2ab (not shown).

Analysis of neural markers expression by immunofluorescent staining in the expansion and differentiation of neural progenitor cells

Immunostaining yields valuable information on progenitor multipotency [15]. Therefore this technique was applied to analyse cells under culture conditions that promote proliferation or differentiation to utilize it as a benchmark technique to monitor cell state for comparison with QRT–PCR results. Staining was conducted with antibodies against nestin (under cell expansion conditions) and β-tubulin III, GFAP and O4 (under cell differentiation conditions), and images were collected by fluorescence confocal microscopy (Figure 2).

Under expansion conditions, we observed that most of the cells express nestin, an intermediate filament protein specifically found in neural stem and precursor cell populations [19]. Subsequently, uncharacterized signals in serum promote progenitor differentiation into glial lineages, and retinoic acid promotes neuronal differentiation [15]. We differentiated cells by culturing them in FBS plus retinoic acid for 8 or 14 days, and during this treatment, cells flattened and extended elaborate neural processes. Subsequent immunostaining demonstrated that the resulting cultures acquire markers of differentiating neurons (β-tubulin III), astrocytes (GFAP) and oligodendrocytes (O4, a cell-surface oligosaccharide epitope), as has been previously shown [15]. Similar results were obtained with an alternate early neuronal marker (Map2ab; images not shown). These results are consistent with earlier reports that progenitors maintain their multipotency when grown in FGF-2 and N2 but express lineage-specific markers under differentiating conditions

Table 1 Fractions of cells expressing various cell lineage markers on day 0 (proliferating conditions) and after 8 days under differentiating conditions, as determined by immunostaining

At least 1000 cells were counted to determine the percentages of cells expressing the markers nestin (immature neural), GFAP (astrocyte), O4 (oligodendrocyte) and Map2ab (neuron).

Day	Marker	Fraction of cells (%)
0	Nestin	100
	GFAP	5.0
	O4	1.6
	Map2ab	0.0
8	Nestin	55
	GFAP	67
	O4	31
	Map2ab	2.1

[6,15]. For comparison with QRT–PCR results, the fraction of cells expressing each marker was manually quantified on days 0 and 8 via confocal microscopy (Table 1). A significant fraction of cells co-expressed nestin and GFAP, likely indicating that they were in the process of undergoing astrocytic differentiation. Analogous quantification on day 14 was prohibitively difficult due to the high density of cells and cellular processes.

Development of the QRT–PCR primers and methods

Although immunostaining of lineage-specific protein markers yields cell-state data, staining slides and manually counting cells of each phenotype is a time- and labour-intensive method, and accurate quantitative results can be difficult to obtain, particularly for high-density neural cultures with dense processes. An alternative method, QRT–PCR, has the potential advantages of being a high-throughput means to accurately quantify the expression of lineage-specific mRNA, though as a downside it provides a population averaged measurement rather than total cell counts of each type.

To evaluate its potential, QRT–PCR using the SYBR® Green dye was developed as a method to assay cell state. Four genes known to be expressed in the process of neural progenitor proliferation and differentiation were selected: nestin, β-tubulin III, GFAP and MBP. To prepare samples for QRT–PCR, total RNA was isolated from cell cultures, and mRNA was reverse-transcribed using an oligo-dT primer.

Since SYBR® Green dye binds to all double-stranded DNA formed during the subsequent PCR, primers were carefully designed with the aid of software (Vector NTI and PubMed BLAST) to enhance amplification specificity. The primers were designed for rat sequences, but could readily be adjusted for the mouse and human homologues. Next, QRT–PCR reactions were optimized as a function of magnesium concentration and primer annealing temperature in order to increase reaction specificity and efficiency. The specificity was confirmed using melting-curve and gel-electrophoretic analysis to confirm the presence of a single band (results not shown). Furthermore, the band was cloned using pCR II (Invitrogen), a T-overhang cloning vector, and the identity of the amplicon was confirmed by DNA sequencing. The following conditions were found to yield high-quality results: 40–50 cycles of 30 s denaturation at 94 °C, 30 s primer annealing at 60 °C (β-tubulin III), 65 °C (GAPDH, GFAP and MBP) or 68 °C (nestin), and 15 s elongation at 72 °C. If specificity had posed a problem, the method could have readily been converted into a TaqMan or molecular beacon approach through the addition of a third fluorescently labelled primer to increase amplification specificity [20].

For quantification of unknown samples, standard curves were generated during every run using linearized pCR II plasmids containing the cloned amplicon as standards. Five serial dilutions were analysed in duplicate for each control. A plot of the threshold cycle at which amplification was detected versus the logarithmic value of the input template concentration was consistently linear (with a typical $R^2 > 0.99$). Control assays were also performed for each set of primers in reactions containing no templates. These assays yielded negligible signal, which suggests that DNA contamination and primer–dimer formation were insignificant factors. Expression of specific mRNA can be normalized to the total amount of RNA added to the RT reaction or to the expression level of a housekeeping gene (such as β-actin, β-2 microglobulin and GAPDH) that may presumably be expressed at the same level from cell to cell. We attempted both approaches, and normalization to total RNA amount provided superior results as compared with GAPDH, perhaps due to cell-to-cell variability in the expression of housekeeping genes under different conditions [21,22]. All results are therefore reported as normalized to total RNA. The list of primer sequences is summarized in Table 2.

Analysis of neural markers expression by QRT–PCR in the differentiation of neural progenitor cells

The QRT–PCR methods were subsequently validated by quantifying the expression of lineage-specific markers during progenitor differentiation. RNA from progenitors cultured under differentiation conditions was collected at different time points over 14 days. Figure 3 (topmost panel) shows that nestin levels progressively increased until day 4 and then decreased, a result likely due to a transient expansion of the neural progenitor population prior to cell differentiation. In contrast, the levels of β-tubulin III, a marker of cell commitment to a neuronal fate, increased by day 4 and then remained approximately constant. This result is consistent with the immunostained cultures (Table 1), but the rapid QPCR (quantitative PCR) method readily facilitates data

Table 2 Oligonucleotide primers that were designed and used in the present study

Marker	Sense primer 5′ → 3′	Product size (bp)	Antisense primer 5′ → 3′
GAPDH	GGTGTGAACGGATTTGGCCGTAT	267	CTCAGCACCAGCGTCACCCCATT
Nestin	CCGGGTCAAGACGCTAGAAGA	196	CTCCAGCTCTTCCGCAAGGTTGT
β-Tubulin III	GTCCGCCTGCCTCTTCGTCTCTA	93	GGCCCCTATCTGGTTGCCGCACT
GFAP	CTCCTATGCCTCCTCCGAGACGAT	170	GCTCGCTGGCCCGAGTCTCTT
MBP	CACAGAAGAGACCCTCACAGCGACA	136	CCGCTAAAGAAGCGCCCGATGGA

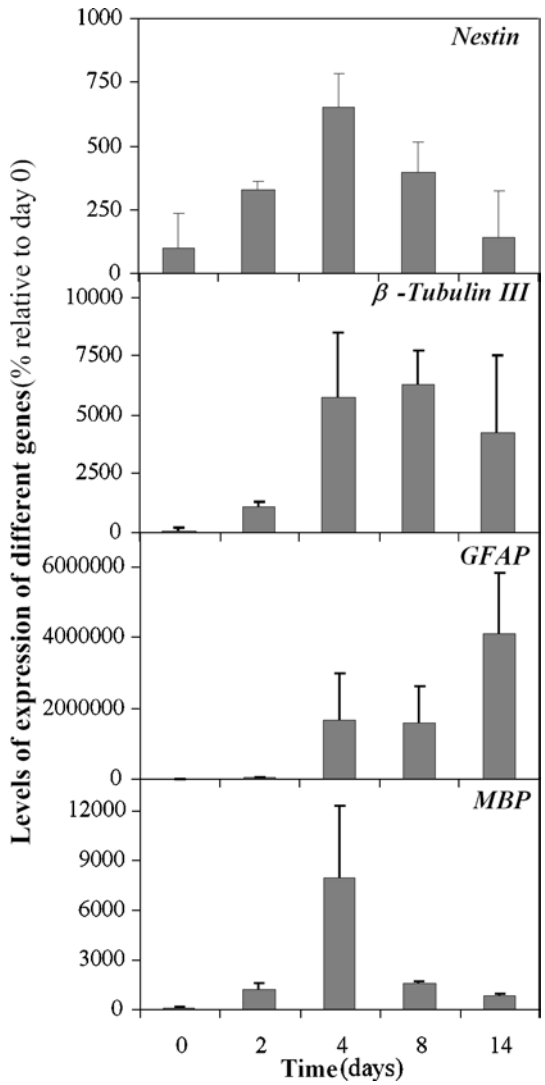

Figure 3 QRT–PCR measurement of the levels of marker gene expression upon cell differentiation for 2 weeks

Nestin expression is an indicator of the relative levels of immature neural cells, whereas the rise in β-tubulin III and GFAP expression represents progressive neuronal and astrocytic differentiation respectively. Finally, MBP expression indicates that oligodendrocyte marker expression initially rises in the population, then decreases in relative proportion compared with the other lineages in the culture. The bars represent the means and S.D. for two biological duplicates that showed similar expression of the control gene (*GAPDH*).

acquisition at multiple time points to yield a dynamic view of cell differentiation.

GFAP expression increased continuously throughout the experiment, indicating that the fraction of cells differentiating into an astrocytic lineage escalates with time. This result is consistent with our immunostaining (Table 1) and with previous reports [15] that indicate that a large percentage of the cells differentiate into astrocytes upon the addition of serum. Finally, oligodendrocytic marker expression increased significantly by day 4 and then decreased. This QRT–PCR result implies that a fraction of cells initially differentiate into oligodendrocytes, but this fraction is soon overtaken by astrocytes and neurons. This result contrasts slightly with the immunostaining results, which indicate a significant increase in the oligosaccharide marker O4 levels from day 0 to day 8. However, although the kinetics of marker expression differ somewhat, both results are consistent with a significant fraction of cells undergoing oligodendrocytic expression.

In summary, the dynamic QPCR measurements, coupled with the endpoint immunostaining results, indicate that nestin-positive cells proliferate until day 4 and then undergo tripotent differentiation.

Furthermore, this pilot experiment establishes QRT–PCR as a valuable technique that can be applied to other studies.

Preliminary QRT–PCR analysis of neural progenitor culture conditions

For long-term expansion, it is important to maintain cells in an immature state. Both to test the expansion conditions, as well as to apply the QRT–PCR methodology to study this problem, we analysed cell state under several growth conditions. RNA was collected at different time points from cells cultured for 4 days. During this time, cells were cultured either without changing the medium (−, unfed cells) or with medium change on day 2 (+, fed cells).

Cell number was counted prior to mRNA isolation (Figure 4a), and it was observed that medium change promoted increased cell counts, presumably due to the renewal of one or more limiting components of the medium that had been depleted. Since analysing cell growth provides only a one-dimensional view of stem and progenitor cell state,

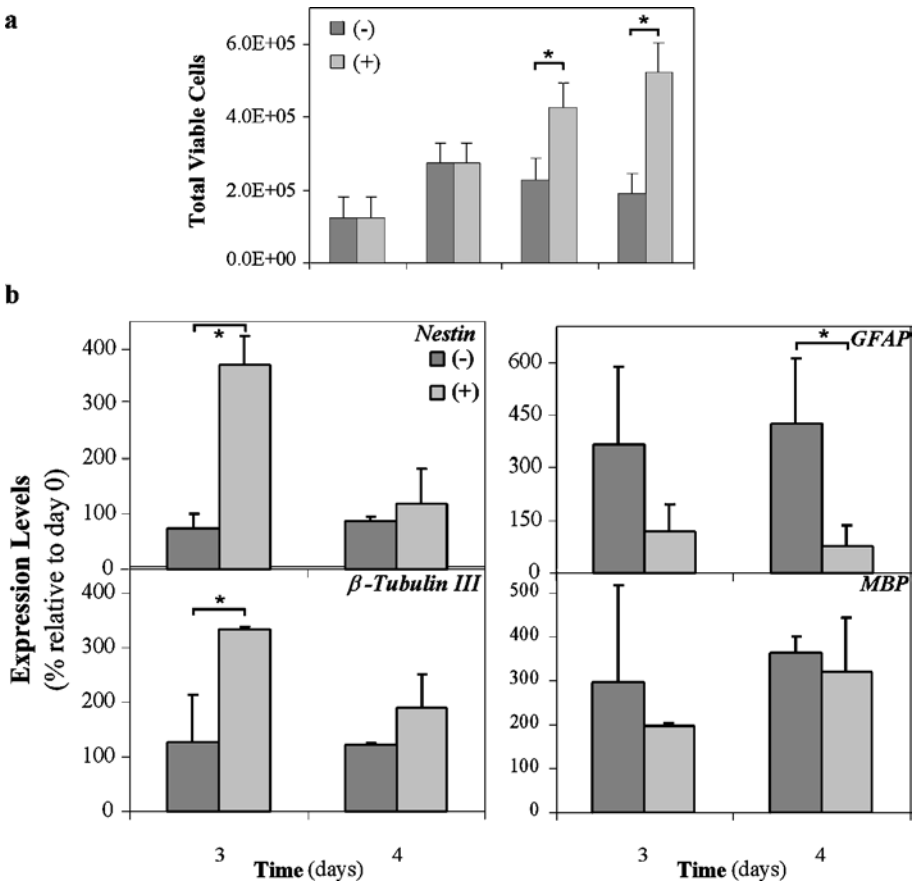

Figure 4 Neural progenitor cells were cultured for 4 days without changing the medium (–) and with medium change on day 2 (+)

The effects of feeding were analysed for (**a**) the total viable cells and (**b**) the expression of differentiation markers. Samples that yielded statistically significant results ($P < 0.05$ in a t test) are denoted with an asterisk. In (**a**), $6.0E+05 = 6 \times 10^5$ etc.

we proceeded to analyse cell differentiation. Figure 4(b) presents the QRT–PCR analysis of cell differentiation obtained for cultures previously fed or not fed on day 2. The marker levels were identical in all samples for the first 2 days (results not shown). However, the levels of nestin and β-tubulin III were subsequently higher for the fed cells, indicating that most of the cells were either maintained in an immature state or slightly biased towards the potentially desirable neuronal lineage. In contrast, the unfed cells experienced a significant increase in GFAP expression and a slight but not significant increase in MBP. This result indicates that a regular feeding schedule is necessary to maintain cells in an immature state and prevent cell bias towards an astrocytic lineage.

Discussion

Stem cells in general, and neural stem cells in particular, have enormous potential for tissue regeneration. However, stem-cell culture for tissue-engineering applications hinges upon the ability to develop robust technology platforms to expand cells in an immature state and subsequently to partially differentiate them towards a specific lineage prior to implantation. At this stage, these efforts should be focused on multiple potential sources of neural stem cells; therefore we are also studying embryonic stem cells and the factors that might be crucial for the scale-up of their growth and differentiation into neural precursors [23]. Regardless of the cell source, a set of rapid, accurate and quantitative tools to analyse cell state will aid in the optimization of growth and differentiation conditions. Accordingly, QRT–PCR methods have been developed here to quantify cell differentiation.

A relatively smaller, but growing, number of methods is available to quantify cell differentiation. Immunostaining has served as the standard molecular tool for analysing cell phenotype [1–4,6,10,11,15,16,24], and the fraction of cells in each differentiated state can be counted in stained cultures. However, at best this is an extremely laborious method, since the elaborate morphologies of most cytoskeletal (GFAP, β-tubulin III and nestin) and cell surface (O4) markers

of cell fate yield complex images whose quantification cannot be readily automated, and large numbers of cells must be counted to yield significant results. Nuclear markers of cell fate could be much more readily quantified via automated image analysis, owing to the comparatively simple shape of a nucleus, but a nuclear marker (NeuN) is commonly available only for neuronal fate [6,10,25].

An alternative powerful method, namely the use of a lineage-specific promoter driving the expression of a reporter gene such as the GFP (green fluorescent protein), enables the analysis of differentiation state in live cells. However, this approach requires the delivery of the promoter–reporter DNA construct to cells, either through the development of transgenic animals or through the use of an efficient gene-delivery vector. At this time, transgenic animals have been developed with one promoter–reporter construct at a time, allowing the analysis of only a single cell fate [26]. In addition, nestin promoter–GFP reporter constructs have been successfully delivered using adenoviral vectors [27], though adenoviral vectors also express numerous viral genes that can perturb the cell state.

With the advent of real-time PCR cyclers, QRT–PCR has emerged as a rapid and accurate means to quantify the expression of specific mRNAs and therefore as a powerful means to quantify stem and progenitor cell culture state. PCR coupled with gel-electrophoretic analysis has been effectively applied to monitor adult neural progenitor differentiation [28], but the typically larger amplicons used for semi-quantitative PCR must be redesigned for QPCR. QRT–PCR has been implemented to monitor the expression of several specific neuronal markers upon the differentiation of the P-19 mouse embryonic carcinoma cell line [29], but neural progenitor, astrocyte and oligodendrocyte fates were not monitored. We report the development of a complete set of QPCR methods to analyse numerous neural cell fates, including neural progenitors (nestin), cells committed to a neuronal fate (β-tubulin III), astrocytes (GFAP) and oligodendrocytes (MBP). These optimized methods have been utilized to analyse cell cultures under conditions that promote differentiation, and the endpoint QPCR results (Figure 3) compare well with immunostaining results (Figure 2).

We next applied QPCR in an initial step to monitor the effects of culture conditions on cell fate. Specifically, as a preliminary and simple study, we began to explore the effects of feeding schedule on cell function. Cells were cultured over a 4 day period, and the medium was changed at the end of day 2 in half of the cultures. Feeding on day 2 maintains high levels of the immature progenitor marker nestin. In contrast, the unfed cells experienced glial differentiation, as indicated by a significant increase for the astrocyte marker GFAP and a moderate increase in MBP. Depletion of a growth factor or nutrient, or accumulation of a metabolic by-product, may be responsible for a transition from cell proliferation to glial differentiation. Future studies should focus on identifying the limiting media component(s).

The methods developed, including standard approaches to measure cell proliferation and viability coupled with a set of QPCR methods to monitor neural cell fate, should prove valuable for numerous applications. First, they should aid in basic studies of signalling mechanisms that regulate neural progenitor function. Additional markers can be added to analyse markers of specific neurotransmitter neuronal phenotypes for higher resolution differentiation information. Secondly, these methods can readily be applied to classic media and culture condition optimization approaches. For example, high-throughput analysis of cell state can be coupled with a Plackett–Burman multifactorial optimization and other biochemical-engineering approaches [30–32] to test the effects of pH, oxygen, growth factors, amino acids and carbon source on cell proliferation and differentiation.

In summary, we have developed QPCR methods to quantify neural stem and progenitor cell fate, a method that yields accurate and rapid cell fate data. Finally, these methods were applied in a preliminary investigation of cell-culturing conditions that showed that fresh N2 medium is necessary to maintain cells in a non-differentiated state. These methods should prove valuable for studying how cellular signalling mechanisms, media composition and culturing conditions modulate and control stem-cell fate decisions.

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