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Variation in neuronal differentiation of a newly isolated mouse embryonic stem cell line: a detailed immunocytochemistry study

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Abstract

Neural precursor differentiation from mouse ES (embryonic stem) cells have been demonstrated using EB (embryoid body), co-culture on stromal feeder layers, and in the absence of external inducing signals. Most of available mouse ES cell original research articles have worked with only six different cell lines. Our goals were to isolate one new mouse ES lineage, and perform a detailed immunocytochemistry study during neural differentiation, making use of an EB strategy protocol following the generation of neural progenitors, glial cells and postmitotic neurons. The dynamics of differentiation of ES cell derived neuronal precursors into differentiated glia cells and neurons were followed *in vitro* and correlated to exposure to specific elements of feeder medium. Morphological aspects of generated cellular types, including its immunocytochemical expression of differentiation markers were studied. Immuno-positivity against β-III tubulin, PGP and TH (tyrosine hydroxylase) was observed from stage I. Approximately 80% of cells were positive for TH at stage I. The first glial cell type appears in stage III. TH, PGP or β-III tubulin-positive cells with neuronal typical morphology only being seen in stage III when TH-positive cells corresponded to approximately 12% of total cells. Variations among other literature findings can be explained by the choice we made to use a newly isolated ES cell line. As colonies may behave differently during neuronal differentiation, it reinforces the necessity of studying original ES cell lines.

Keywords: embryonic stem cell differentiation; immunocytochemistry; neural differentiation; neural precursor

1. Introduction

Although stem cell research has become one of the important fields in biomedical sciences, knowledge of the mechanisms regulating ES (embryonic stem) cell differentiation is still limited to a few signalling pathways and regulatory factors (Wobus and Boheler, 2005). The key challenge in using ES cell progeny for cell replacement therapies in Parkinson's disease is the generation of neurons expressing the complete transcription, biochemical and functional profile of a mature midbrain dopamine neuron *in vitro* and *in vivo*.

There are considerable differences in the strategies used to achieve neural induction among the available protocols. Most of them can be grouped into three main categories: (a) EB (embryoid body)-mediated differentiation, (b) differentiation induced via co-culture on stromal feeder layers and (c) neural induction through 'default' in the absence of inducing signals (McGrew et al., 1995; Bang et al., 1997; Kriks and Studer, 2009).

It is known that prolonged *in vitro* cultivation of ES cells can cause cell modifications and that different lineages may behave differently during cell differentiation, thus needing a larger number of studies on new SC lines using samples that have undergone a few passages. Most of the literature between 1981 and 2001, worked with only six cell lineages (Downing and Battey, 2004).

Our goals in this work were to carry out a detailed immunocytochemistry study of mouse ES cell neural differentiation following the generation of neural progenitors, glial cells and postmitotic neurons through an EB-mediated differentiation protocol using a newly isolated mouse ES lineage.

2. Materials and methods

2.1. ES cell culture and PCR

Our research was approved by The Ethics Committee (CETEA-UFMG, protocol 04/2006). A mouse ES cell line named CT-4 was isolated according to previous publications (Nagy et al., 2003; Tavares et al., 2007). Briefly, blastocysts from 129S1/Sv mice were cultured in 24 well dishes prepared with 0.1% gelatin and

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primary mouse embryonic fibroblasts (Specialty Media). Each litre of ES cell medium consisted of 750 ml of DMEM (Dulbecco's modified Eagle's medium), FBS (fetal bovine serum) (20%), 2 mM L-glutamine, 50 µg/ml pen/strep (penicillin/streptomycin), 0.1 mM MEM NEAA (non-essential amino acids), 10 ml nucleosides, 0.1 mM 2-mercaptoethanol (all from Specialty Media) and 2000 units/ml LIF (leukaemia inhibitory factor) (Chemicon International). The medium was changed daily for 6 days.

Every 2–3 days these cells were submitted to trypsin treatment. Between the fifth–seventh passages the undifferentiated state was confirmed by expression of Nanog (For-AGGGTCTGCTACTGA-GATGCTCTG/Rev-CAACCACTGGTTTTTCTGCCACCG) and Oct-4 (For-GGCGTTCTCTTTGGAAAGGTGTTC/Rev-CTCGAACCACATC-CTTCTCT) genes by PCR.

Tubes were incubated in a thermal cycler at 94°C for 1 min. Forty cycles of PCR amplification were performed as follows: denaturation at 94°C for 30 s, annealing at 55°C for 39 s and extension at 72°C for 53 s (Tavares et al., 2007). RT–PCR (reverse transcriptase–PCR) and PCR were run with negative controls and 10 µl of PCR products were analysed by 2% agarose gel electrophoresis.

2.2. Neuronal differentiation protocol

Samples of newly isolated ES cell were kept in liquid nitrogen. Thawed samples at the fifth–seventh passages were used for neural differentiation, which was achieved in five stages, as described in previous publications (Okabe et al., 1996; Lee et al., 2000; Nishimura et al., 2006) with modifications.

At stage I ES cells were expanded in medium culture containing Knockout DMEM, 15% FBS, 2 mM L-glutamine, 50 µg/ml pen/strep, 0.1 mM NEAA (all from Invitrogen), 0.1 mM 2-mercaptoeth-anol (Millipore) and 1400 units/ml of LIF (Sigma–Aldrich) in 0.1% gelatin. The stage II generated EB. After dissociation, 5.32×10^5 cells were cultured in suspension in 60 mm plates for 4 days in standard culture medium (4 ml) without FBS and LIF. KOSR (knockout serum replacement) was used instead of FBS.

In stage III, the EB were cultured for 1 day in standard culture medium containing FBS, and then the medium was replaced with ITSFn (insulin–transferrin–selenium–fibronectin) medium. After 6 days, cells were dissociated (stage IV) and plated at 1.5×10^5 cells/cm^2 in polyornithine (15 µg/ml)/laminin (1 µg/ml) substrate for 6 days. The stage IV medium contained DMEM/F12, 1% N2 medium, 1 µg/ml laminin (all from Invitrogen), 10 ng/ml bFGF (basic fibroblast growth factor), 500 ng/ml SHH-N and 100 ng/ml FGF-8b (fibroblast growth factor 8b; all from R&D Systems).

Stage V medium contained DMEM/F12, 1% N2 medium, 1 µg/ml laminin, 1% pen/strep and 200 µM ascorbic acid (MP Biomedicals). The culture was maintained in differentiation medium for 6 days. ES cells were submitted to prolonged culture (26 days).

2.3. Immunocytochemistry

ES cells, well-known neural precursors and neuronal markers, were studied by immunofluorescence at each stage. Fixation and immunocytochemical analysis of cells were performed as described earlier (Seaberg and van der Kooy, 2002). Briefly, the cells were plated on 24 wells containing glass coverslips,

previously treated with polyornithine. At the end of this period, namely 1 day for stage I, 3 days for stage II and 6 days for the stages III–V, the coverslips were washed with PBS, fixed with formalin 10% in PBS, and stored for weeks to months at 4°C.

Primary antibodies used, included anti-SSEA-1 (stage-specific embryonic antigen 1) mouse monoclonal (1:250; Santa Cruz Biotechnology), anti-Oct-3/4 rat monoclonal (1:50; R&D Systems), anti-GFAP (glial fibrillary acidic protein) rabbit polyclonal (1:500; Dako Glostrup), anti-nestin rabbit polyclonal (1:1000; Abcam), anti-β-III tubulin rabbit polyclonal (1:480; Abcam), PGP 9.5 rabbit polyclonal (1:600; UltraClone Ltd), and anti-TH (tyrosine hydro-xylase) rabbit polyclonal (1:300; Millipore Corporation).

Secondary antibodies Alexa Fluor® 488 goat anti-mouse IgG, anti-rat IgG, or anti-rabbit IgG; Alexa Fluor® 546 goat anti-rabbit IgG were all from Molecular Probes, Invitrogen. Secondary antibody-only wells were processed simultaneously using the identical protocol, except that solutions did not contain primary antibodies. All secondary-only controls were negative for staining. Cell nuclei were counterstained with 0.2 µg/ml Hoechst (Invitrogen) for at least 5 min at room temperature, then washed thrice in PBS at 10% in distilled water and mounted with hydromount (National Diagnostics).

Fluorescent images obtained under an Olympus BX51 (Olympus) microscope were transferred via video camera Cool SNAP-Procf Color (Media Cybernetics) to a video system using the Image-Pro Express version 4.0 for Windows (Media Cybernetics). The recorded images were used for counting cells stained by specific antibodies and nuclei in total. The representative images of the expression of different markers in the stages studied were selected and prepared as Figures using Photoshop 6.0 (Adobe) software.

2.4. Quantification

We captured 8–30 fields per coverslip at magnifications of $\times 20$ and $\times 40$, depending on the stage of differentiation, which directly affects the density of cells per field. We obtained at least 1000 cells counted per coverslip by evidence of their Hoechst stained nuclei. The result was expressed as the mean ratio between the number of cells positive for the antibody under study and the number of nuclei counted per image $\pm$ S.E.M. For each antibody at each stage the counting procedure was performed in 3–5 coverslips, derived from at least three repeated differentiation experiments.

2.5. Statistical analysis

Data is expressed as means $\pm$ S.E.M. unless specified otherwise. Statistical comparisons of expression of each antibody at different times of differentiation were performed using the Friedman test with Dunn's multiple comparison test. The acceptable level of significance was considered at $P < 0.05$.

3. Results

3.1. Morphological characterization of cells

Undifferentiated ES cells exhibited the expression for SSEA-1 (75%, $n = 1000$ cells) (Figure 1A), in correspondence with RT–PCR
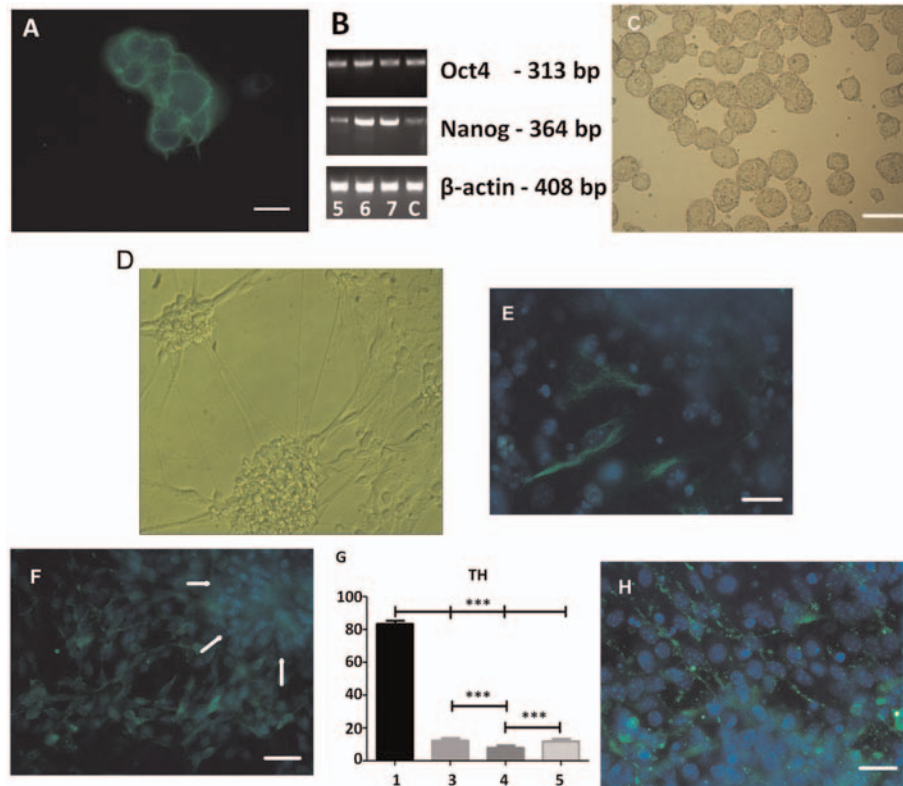
Figure 1   Analysis of morphology and marker expression in mouse ES derived progenitor cells
(**A**) SSEA-1 immunostaining of stage I cells counterstained by Hoechst. (**B**) RT–PCR analysis of cells at stage I for Nanog and Oct4 genes. Panels 5, 6 and 7 show 2% agarose gels stained with ethidium bromide for the 5th, 6th and 7th passages, respectively; (**C**) is a positive control with one zona pellucida free blastocyst. (**C**) Phase contrast micrograph of EB at Stage II. (**D**) Phase contrast micrograph of a sphere cultured in serum-free medium at Stage III. (**E**) GFAP indirect immunofluorescence of accessory cell at stage III. (**F**) PGP immunopositive 'rosette' (white arrows) at stage IV. (**G**) Percentage of TH+ cells in subsequent stages (I, III–V) of differentiation derived from three independent experiments; ***$P < 0.0001$ by Friedman test and Dunn post-test. (**H**) Increased complexity of neurites immunostained for β-III tubulin. Scale bars $= 20$ μm (**A**, **D**, **E**, **F**, **H**), 200 μm (**C**).

for Nanog and Oct-4 (Figure 1B). At stages I and II, small and sparse PGP immunoreactive cells were found, mostly located inside conglomerates. These cells did not express TH and GFAP. At this point also nestin-positive cells were present at large amounts (78%, $n = 1000$ cells). Cultured stage I cells proliferated and formed large and spherical floating aggregates known as EB (Figure 1C). Almost half of plated cells adhered to the bottom and consistently expressed PGP by immunocytochemistry despite being TH-negative (data not shown). The cell clusters derived from modified EB are called neurospheres and form a network that becomes more evident in subsequent stages (Figure 1D). At the end of 6 days of cultivation in this medium, many extensions of cytoplasm of individual cells are highly visible, and connecting EB are often supported by a flat monolayer of mostly GFAP-positive cells (Figure 1E).

The formation of neurospheres remained after dissociation, between stages III and IV. In the spaces between the neurospheres, we observed rosette-like structures (Figure 1F). Cells that migrated from neurospheres had triangular/polygonal morphology, convex surfaces and protruded above the plane of the substrate. They were similar to neurons, emitting thin extensions called neurites which connect one cell to another and sometimes

present a structure resembling a 'rosary bead', detail easily visible when stained by β-III tubulin, PGP and TH.

There were well-differentiated neuronal bodies aligned like a fence connecting the neurospheres and superposed to large cells of mesenchymal appearance, subsequently identified as glia cells by GFAP staining. At stage V, the overall appearance remained similar to the previous stage, but the presence of apoptosis figures became more frequent. There were significantly increased numbers of PGP immunopositive cells both with typical neuronal morphology and great neurites complexity organized in 'rosette' formations (Figure 1H, white arrows).

3.2. Quantitative assessments

Stage I was positive for SSEA-1 and Oct-4. At stage III, neurospheres presented cells strongly stained by PGP exhibiting neuronal soma and some neurites. The number of these cells was increased further at stages IV and V. GFAP and TH were expressed from stages III–V. The percentages of TH+ cells in subsequent stages (I, III–V) of differentiation derived from 3 separate experiments are illustrated in Figure 1(G). The percentage of positive cells stained by phenotypic markers of undifferentiation (SSEA-1) and

cell differentiation (β-III tubulin, PGP and TH) were determined at each stage of our protocol and all results are expressed in Figure 2. All markers decreased significantly from stage I to the other stages ($P<0.0001$). SSEA-1 and Nestin decreased significantly from stages III–IV to IV–V ($P<0.0001$). PGP and tubulin immunopositive cells percentage did not vary between stages III and V (Figure 2).

4. Discussion

In recent decades, numerous studies have been developed in order to obtain functional cells from ES cells (Ohse et al., 2009). ES cell culture without LIF or exposure to retinoic acid promotes neural differentiation of EB efficiently, and Lee et al. (2000), refined this method to induce serotonergic and dopaminergic neurons from ES cell neural precursors.

Our protocol modified previous protocols (Okabe et al., 1996; Lee et al., 2000; Nishimura et al., 2006), using KOSR instead of FBS in stage II in order to control inhibitory signalling of neuronal differentiation. A period of 25 days was needed for this transformation. The percentage of positive cells stained by phenotypic undifferentiation (SSEA-1, Nestin) and cell differentiation markers (β-III tubulin, PGP and TH) at each stage of our protocol showed statistically significant differences from stage I to the other stages. Undifferentiated (SSEA-1) and neural precursors (nestin) markers progressively reduced expression over the days of culture, as expected.

The immuno-positivity against β-III tubulin, PGP and TH was observed from stage I and may indicate a standard 'default' differentiation to neuronal cells. The first glial cell type, the astrocyte (GFAP-positive), appeared in stage III and it was not present at stage I, indicating that the neuronal default behaviour

does not extended to these accessory cells before LIF is withdrawn.

The generic marker of neuron status (PGP) and dopaminergic differentiation (TH) remained virtually stable. Differently from Lee et al. (2000), who found TH-positive cells at stage V, we found approximately 80% of cells positive for TH at stage I, but they presented undifferentiated morphology (Figure 1G). TH, PGP or β-III tubulin-positive cells with neuronal typical morphology were only seen in stage III, when TH-positive cells corresponded to approximately 12% of total cells. In stage V, Lee et al. (2000) found 70% of TUJ1-positive cells (similar to β-III tubulin) of which approximately 7% were TH-positive.

The uniform expression of nestin-positive cells in stage IV, although not quantified, indicates that these proliferating cells already committed to neural lineage may be the source of the TUJ1 cells and mesencephalic TH neurons found by Lee et al. (2000), while our source of nestin-positive cells were drastically reduced by stage III, resulting in approximately 10% of nestin-positive, 8% of β-III tubulin-positive, 21% of PGP-positive and 12% of TH-positive cells. Nishimura et al. (2006) found approximately 5% of nestin-positive cells, 32% of TH-positive, 80% of microtubule-associated protein-2-positive and 10% of GFAP-positive in stage V, but the dynamics of the differentiation process cannot be discussed because they do not present quantified cells in the previous stages.

Our approach was able to distinguish the early phases of neurogenesis, including pluripotent ES cells, the proliferation of neural progenitors, and their differentiation into neurons and glial cells. As we did not use one ES line from previous studies, we cannot precisely show how different the differentiation is to these lines. We also have not yet invested in assays required to demonstrate neuronal and dopaminergic function. Electrophysiological measurements should be used comparing physiological
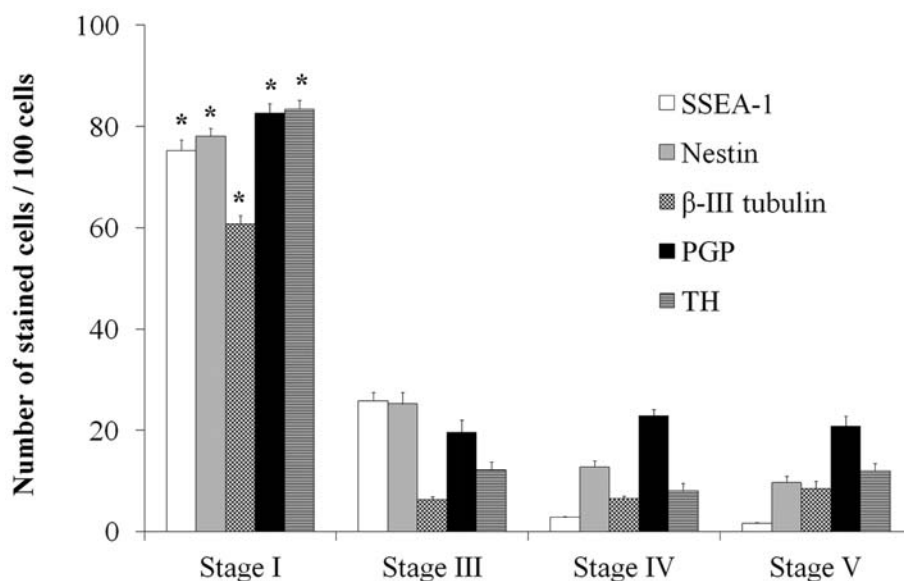

Figure 2   **Percentage of positive cells stained by phenotypic markers of undifferentiation (SSEA-1, nestin) and cell differentiation (β-III tubulin, PGP and TH) at each stage of our protocol**
*Indicates statistically significant differences from stage I to the others ($P<0.001$).

behaviour of ES cells derived (Kim et al., 2002; Barberi et al., 2003; Perrier et al., 2004) to primary midbrain dopamine neurons (Grace and Onn, 1989). Biochemical function can be assessed *in vitro* by measuring DA release via HPLC analysis (Lee et al., 2000, Barberi et al., 2003; Perrier et al., 2004) or *in vivo* via microdialysis (Rodriguez-Gomez et al., 2007).

In conclusion, this work is aligned to other *in vitro* models allowing the dissection of cellular and molecular processes accompanying the various stages of development of the mouse nervous system. To the best of our knowledge this detailed immunocytochemistry study using all the related methods has not been reported yet. Variations among other findings in the literature can be explained by the choice we made to use a newly isolated ES cell line, and given that colonies may behave differently during neuronal differentiation, it reinforces the necessity of studying original ES cell lines. Ideally this protocol should be a tool to further study the regulatory mechanisms involved in the sequential differentiation steps of diverse types of neurons.

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