Characterization of P19 Cells during Retinoic Acid Induced Differentiation

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Abstract: The aim of our study was to characterize mouse embryonal carcinoma (EC) cells P19 in different stages of retinoic acid induced neurodifferentiation by two methods, immunocytochemistry and RT qPCR. The characterization of the cells is crucial before any transplantation into any model, e.g. in our case into the mouse brain with the aim to treat a neurodegenerative disease. Specific protein markers (MAP-2, OCT-4, FORSE-1) were detected by immunocytochemistry in the cell cultures. The mRNA expression levels of PAX-6, MASH-1, Brachyury,

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GATA-4 and AFP were determined by RT qPCR method. HPRT was used as a housekeeping gene. The degree of differentiation can be characterized by expression of analyzed genes. The presence of OCT-4 and FORSE-1 proteins in undifferentiated pluripotent cells and the presence of dendrite specific MAP-2 in neuroprogenitors was detected. The expression levels of PAX-6 and MASH-1 increased and expression of Brachyury decreased during the neurodifferentiation process. The expression levels of GATA-4 and AFP were the highest after induction of differentiation with retinoic acid. Detailed characterization of cells before transplantation experiments can contribute to better understanding of their effect.

Introduction

Embryonic stem (ES) cells are pluripotent cells having the ability to differentiate into any cell type. The stem cells allow the body to create new cells and repair the damaged parts of tissues and organs. Stem cells have the capacity not only to differentiate but also the ability of their own recovery. ES cells represent under physiological conditions a universal stock of spare parts for a variety of tissues and organs (Rolletschek et al., 2010). *In vitro* experiments offer a modality to study cell differentiation and function under supervised conditions, with the additional advantage of investigating how interactions between populations of cells influence cell growth and behaviour (Stewart et al., 2004). In our study we used the pluripotent murine embryonal carcinoma (EC) P19 cell, whose developmental potential is similar to embryonic stem cells. These cells can differentiate into cell types of all three germ layers – ectoderm, mesoderm and endoderm.

P19 EC cells were derived from a teratocarcinoma of C3H/He mouse strain (McBurney and Rogers, 1982) and can serve as a model for studying differentiation processes. Like other EC and ES cells, P19 cells are developmentally pluripotent and appear to differentiate using the similar mechanisms (McBurney, 1993).

A number of P19 cells characteristics make them a valuable model for studies of early developmental events. The cells are immortal allowing the creation of almost unlimited amounts of material for analysis. They are easy to grow and maintain in the undifferentiated state but they can also be efficiently induced to differentiate by simple manipulation of the culture conditions.

Retinoic acid (RA) can induce P19 EC cells to differentiate into neural cells and this cell system has been shown to be a useful tool for studying the early steps of neural differentiation *in vitro* (Osanai et al., 2003; Hong et al., 2008).

Stem cells therapies can potentially help in the treatment of degenerative diseases. However, before use of the cells it is necessary to understand the behaviour of cells in their niche. Neural differentiation is controlled by complicated molecular mechanisms that determine cell fate and diversity within the nervous system. In clinical practice, the neurotransplantation has been used in the treatment of Parkinson's disease (Björklund and Lindvall, 2000). Although the treatment of

other CNS diseases with transplantation of neuroelements is still at the research stage, the treatment of other neurological diseases has already been tested in human patients. E.g. spinal cord injuries (Sykova et al., 2006), Huntington's disease (Bachoud-Lévi et al., 2000) and strokes (Kondziolka et al., 2004). In our study, P19 EC cells were used to produce differentiated neuronal progenitors as well as differentiated neuron-like cells after *in vitro* induction.

For immunofluorescence assay, antibodies against OCT-4, FORSE-1 (forebrain surface embryonic antigen-1, identical with SSEA-1LewisX/CD15 antigen) and MAP-2 (microtubule-associated protein-2) were used.

Transcription factor OCT-4 is a marker of pluripotency (zur Nieden et al., 2006).

The FORSE-1 is structurally related to SSEA-1 (stage-specific embryonic antigen-1). It is expressed in the inner cell mass (ICM), ES cells and developing mouse brain, where it labels proliferative cells in the embryonic forebrain and in proximity to the central canal (Pruszak et al., 2007).

The MAP-2 is a dendrite-specific protein found specifically in dendritic branching of neuron. It is one of the earliest markers specific for postmitotic neurons (Torii et al., 1999).

For the characterization of the cells by quantitative reverse transcriptase real-time PCR (RT qPCR), the gene markers PAX-6, MASH-1, Brachyury, GATA-4 and alpha-fetoprotein (AFP) were used.

PAX-6 (paired box gene 6) is one of the key factors for the patterning of the CNS and for the differentiation of cortical glial cells. It is a transcriptional factor expressed with a region-specific pattern that plays important roles in CNS development, including brain patterning, neuronal specification, neuroblast migration, axonal projection, and eye development. In the absence of a functional PAX-6, several abnormalities have been detected in the developing cortex, such as failure of neuronal cell migration and altered neuronal cell adhesion (Aronne et al., 2008; Fotaki et al., 2008).

MASH-1 has been found to be expressed in the developing PNS and plays an essential role for the development of autonomic neurons (Torii et al., 1999).

The transcription factor GATA-4 is regarded as marker that is expressed in endoderm- and mesoderm-derived lineages in embryonal bodies (Koike et al., 2007) and reflects endodermal differentiation of pluripotent stem cells.

AFP is the first alpha-globulin to appear in the blood of vertebrates during ontogenesis. The primary roles of AFP are to ensure specific transport and the modulation of the activities of a number of ligands. These ligands, such as retinoic acid, bilirubin, serine protease inhibitor are essential for the differentiation of embryo organs and for foetal development (Lafuste et al., 2002).

The Brachyury (from the Greek "brakhus" meaning short and "oura" meaning tail) gene encodes a transcription factor essential for the genesis and maintenance of mesoderm and notochord (Kispert and Hermann, 1993; Ramirez et al., 2006).

Material and Methods

P19 cell culture and differentiation

The P19 EC cells were purchased from the European Collection of Cell Culture, Wiltshire, UK.

P19 undifferentiated cells were routinely grown at 37 °C, in a humidified atmosphere of 5% CO_2, in propagation medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin (all GIBCO BRL, Chemos CZ, Prague, Czech Republic). Cells were seeded onto gelatine-coated tissue culture dishes and passaged as needed (Pachernik et al., 2005; Hong et al., 2008; Solari et al., 2010).

For neurodifferentiation experiment the cells were cultured in DMEM/F12 (1:1) media supplemented with the mixture of insulin, transferrin, selenium and antibiotics under serum-free conditions. To induce the neurogenesis the cells were treated from day 0 to day 2 (D0–D2) with 5×10^{-7} M RA (all-trans retinoic acid, Sigma, Prague, Czech Republic). Differentiation medium was replenished at D2 (Pachernik et al., 2005).

The characterization of cell populations was performed by RT qPCR and immunofluorescence assay. The samples of undifferentiated (naive) cells (sample D0), neuroprogenitors cultured 3 days with neurodifferentiation protocol (sample D3) and the cells after 10 days of neurodifferentiation protocol (sample D10) were studied. Cells of each sample dish (D0, D3, D10) were stored in 1.5 ml Eppendorf tube at –80 °C until analysis. All samples were analyzed in doublets and average value was calculated.

Indirect immunofluorescence (IIF)

The cells cultured with the same protocol as described above but on the special glass plate were fixed in 4% formaldehyde for 30 minutes on ice and permeabilised in Triton X 100 – 0.2% with Nonidet – NP 40 – 0.5% for 10 minutes. The cells were then quenched with 1% FBS in PBS for 10 minutes and incubated with the

Table 1 – Antibodies used for immunocytochemistry

EC cells	Primary antibodies	Dilution	Secondary antibodies	Dilution
undifferentiated	Hybridoma FORSE-1 (Developmental Studies Hybridoma Bank, University of Iowa)	1:1	anti-mouse IgG+IgM (Invitrogen)	1:750
	OCT-4 (Santa Cruz Biotechnology, Inc.)	1:200	anti-mouse IgG Alexa Fluor 568 (Invitrogen)	1:200
differentiated	MAP2 (SIGMA-ALDRICH)	1:500	anti-mouse IgG (SIGMA-ALDRICH)	1:150

appropriate primary (overnight at 4 °C) and FITC conjugated secondary antibodies (10 minutes at room temperature). The specimens were mounted to DAPI/Antifade solution and viewed under an epifluorescent microscope Olympus BX 41. The following primary antibodies were employed: mouse monoclonal antibody against MAP-2 (M4403, Sigma), rabbit polyclonal antibody against OCT-4 (sc-9081, Santa Cruz Biotech), and mouse monoclonal antibody against Lewis X antigen (FORSE-1, developed by Dr. Patterson, obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences – Iowa City, IA). The dilutions used for detection are shown in Table 1.

Quantitative reverse transcriptase real-time PCR (RT qPCR)

Total RNA was isolated from each collected sample by the Fast RNA Pro Green Kit (Q-BIOgene, Irvine, CA, USA). Reverse transcription (RT) was performed from 3 μg of total RNA with Superscript III Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA) and oligo $d(T)_{21}$ as a primer. The sequences of the primers used for mRNA quantification are shown in Table 2 (Esner et al., 2002; Hong et al., 2008). Primers were synthesized by GeneriBiotech (Hradec Králové, Czech Republic). A quantitative estimation was performed using iCycler apparatus (Bio-Rad, Prague, Czech Republic). As a housekeeping gene, hypoxanthine phophoribosyltransferase (HPRT) was used. The HPRT gene is reported to be constitutively expressed (Pernas-Alonso et al., 1999). HPRT RNA levels are very low, 1 to 10 molecules per cell (Steen et al., 1990), which makes it suitable as an endogenous mRNA control in RT qPCR for quantification of low copy mRNAs (Sellner and Turbett, 1996; Rey et al., 2000; Specht et al., 2001). For verification of the specificity, melt curve analysis was used. PCR products of the quantification were separated on 1.5% agarose gels and visualized by ethidium bromide staining. The results of expression were obtained as a relative copy number (R) using the Ct value of the measured mRNA and Ct value of the normalizer (housekeeping gene, HPRT) by the $2^{-\Delta Ct}$ approach: $R = 2^{-(Ct\ measured\ mRNA\ -\ Ct\ normalizer)}$ (Koike et al., 2007).

Results

Immunofluorescence staining confirmed the presence of OCT-4 and FORSE-1 in undifferentiated cells (Figures 1 and 2) and the presence of dendrite specific protein MAP-2 in neuroprogenitors (Figure 3).

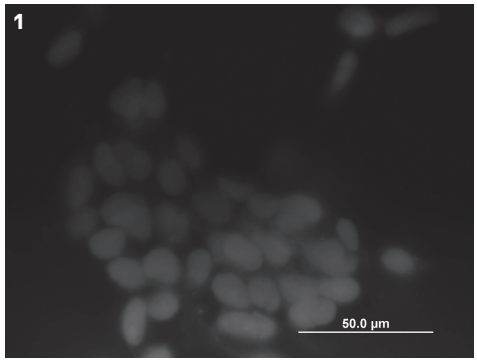
Expression levels of PAX-6, MASH-1, Brachyury, GATA-4 and AFP were determined by quantitative RT qPCR. Example record of PAX-6 quantification is shown in Figure 4.

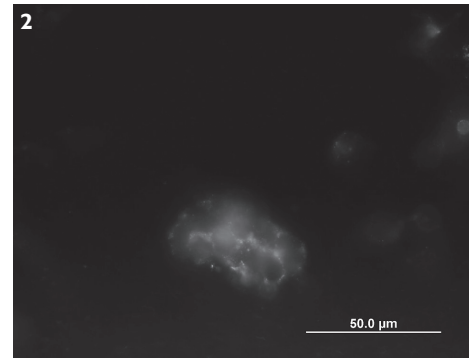
The obtained C_t values and relative copy numbers (R) are shown in Table 3.

For easier comparison of the levels of expression in different stages of differentiation, the relative values are recalculated in Table 4 and shown in Figure 5.

These relative values are calculated as the ratio of the appropriate value to the lowest expression value of the given marker from the estimated stages (D0, D3 and D10). So the value of the lowest expression is set to 1.

The expression levels of PAX-6 and MASH-1 increased and expression of Brachyury decreased during the neurodifferentiation process. The expression levels of GATA-4 and AFP were the highest after induction of differentiation with retinoic acid.





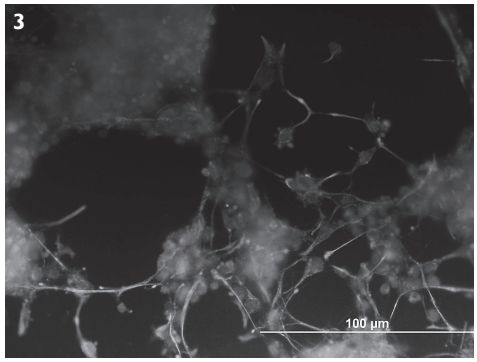


Figure 1 – Immunostaining of OCT-3/4, undifferentiated cells (D0).

Figure 2 – Immunostaining of FORSE-1, undifferentiated cells (D0).

Figure 3 – Immunostaining of MAP-2, differentiated cells after induction with RA (D10).

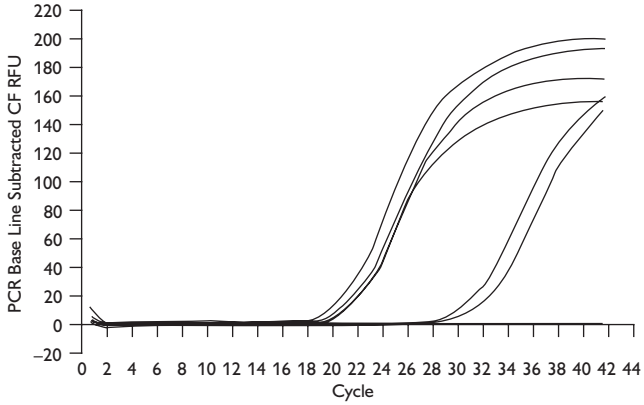


Figure 4 – Amplification curves of the PAX-6 marker (in doublets).

Discussion

To be able to evaluate results and understand the mechanisms of any transplantation experiments, detailed characterization of transplanted cells is needed. The P19 cells in different stages of differentiation were characterized by immunocytochemistry and RT qPCR as well.

Table 2 – Primers used for gene expression analysis

Gene	Primer		Length (bp)
PAX-6	forward	5'-TGCCCTTCCATCTTTGCTTG-3'	178
	reverse	5'-TCTGCCCGTTCAACATCCTTAG-3'	
MASH-1	forward	5'-CTCGTCCTCTCCGGAACTGATG-3'	303
	reverse	5'-CGACAGGACGCCCGCCTGAAAG-3'	230
Brachyury	forward	5'-GAGAGAGAGCGAGCCTCCAAAC-3'	
	reverse	5'-GCTGTGACTGCCTACCAGAATG-3'	
GATA-4	forward	5'-GAAAACGGAAGCCCAAGAACC-3'	186
	reverse	5'-TGCTGTGCCCATAGTGAGATGAC-3'	
AFP	forward	5'-ATGTATGCCCCAGCCATTCTGTCC-3'	466
	reverse	5'-GAGATAAGCCTTCAGGTTTGACGC-3'	
HPRT	forward	5'-CTTGCTGGTGAAAAGGACCTCTC-3'	350
	reverse	5'-CAAATCAAAAGTCTGGGGACGC-3'	

Table 3 – Results of mRNA quantification – C_t values and relative copy numbers (R)

Gene	Day	Average C_t	R
HPRT	D0	19.15	–
	D3	19.55	–
	D10	19.70	–
Pax-6	D0	32.30	0.00011
	D3	22.50	0.12941
	D10	21.55	0.27739
Brachyury	D0	21.70	0.17076
	D3	29.65	0.00091
	D10	32.75	0.00012
Mash-1	D0	29.75	0.00064
	D3	29.00	0.00143
	D10	26.85	0.00704
GATA-4	D0	29.75	0.00064
	D3	29.00	0.00143
	D10	34.15	0.00005
AFP	D0	36.45	0.00001
	D3	25.50	0.01618
	D10	29.90	0.00085

Table 4 – Results of mRNA quantification – recalculated relative values (maximum of expression in bold type)

Gene	D0	D3	D10
PAX-6	1	1176	**2521**
MASH-1	1	2	**11**
Brachyury	**1448**	8	1
GATA-4	14	**32**	1
AFP	1	**2610**	137

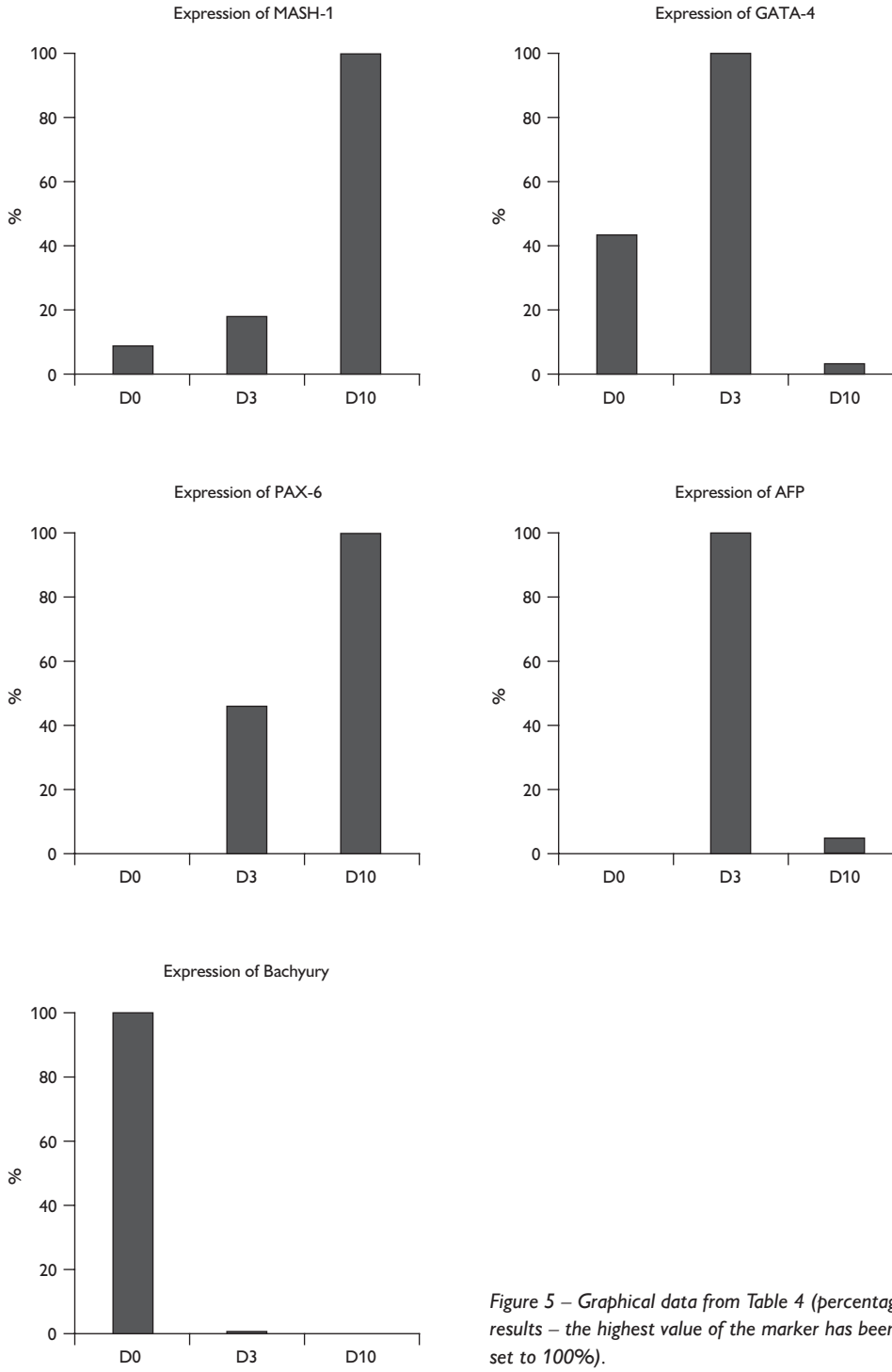


Figure 5 – Graphical data from Table 4 (percentage results – the highest value of the marker has been set to 100%).

By indirect immunofluorescence, the presence of OCT-4 and FORSE-1 was recorded in undifferentiated cells, what confirmed their pluripotency. The presence of dendrite specific protein MAP-2 was found in neuroprogenitors. Using RT qPCR method, the increasing expression of PAX-6 during the neurodifferentiation process was demonstrated.

These findings are in agreement with previously published results that have shown PAX-6 as essential factor for the differentiation of progenitor cells into neurons (Bauwens et al., 2008).

The increase of MASH-1 expression during the neurodifferentiation in our experiments was recorded with accordance with previously published results as well (Kusakabe et al., 2002; Yang et al., 2008).

The Brachyury marker is linked with pluripotent state of cells (Ramirez et al., 2006). The expression of Brachyury in our experiment was high in undifferentiated cells, which are pluripotent. The value of expression rapidly decreased after the induction with RA.

GATA-4 is a tissue-specific, retinoic acid-inducible and developmentally regulated transcription factor. On the basis of its tissue distribution, GATA-4 plays a role in gene expression.

It has been published that GATA-4 mRNA is not detectable in undifferentiated cells but has been induced during differentiation into parietal or visceral endoderm (Arceci et al., 1993). However, we detected GATA-4 expression not only after induction with retinoic acid, but also in undifferentiated cells. This difference could be caused by different methods of estimation – the quantitative RT PCR method in our study, whereas Arceci's results obtained qualitatively by Northern blot.

AFP expression strongly increased after the induction of neurodifferentiation. It is known, that AFP is silent in pluripotent stem cells and activated during differentiation of endoderm. Recently it was published that retinoic acid has an effect on rise of AFP expression mediated by Forkhead family member FOXA-1 (Taube et al., 2010), which could be the explanations of our findings.

Embryonic stem cells are a promising source of differentiated oligodendrocytes and motoneurons and could be used to treat neurological disorders and traumas. Clinical applications of stem cells critically depend on their ability to differentiate toward defined and purified neural cell types *in vitro* (Ronaghi et al., 2010). Our study contributes to optimalization of methods that can be used for EC as well as ES cells characterization. Knowing the optimal way how to truly characterize cells will contribute to better understanding of their qualities and safer use in cellular therapy.

In conclusion, this study showed that PAX-6 and MASH-1 are easily quantifiable markers of RA-induced cell neurodifferentiation process. Assay of these markers is valuable for the characterization of the cells necessary before their transplantation in any neurologic disease and trauma.

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