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IN VITRO DIFFERENTIATION OF THE IMMORTALIZED MESENCEPHALIC PROGENITOR CELL LINE CSM14.1 OCCURS INDEPENDENTLY FROM HAEMATOPOIETIC CYTOKINES

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Abstract – In vitro expanded neural precursor cells could provide a renewable source of dopaminergic (DAergic) neurons for cell replacement therapy. In the present study immortalized cell line CSM14.1 was investigated in vitro. Cells were derived from the ventral mesencephalic area of a 14-day-old rat embryo and immortalized retrovirally with the temperature-sensitive mutant of the SV40 Large T-antigen. We investigate the proliferation and differentiation of these cells under various culture conditions, at different temperatures and serum conditions. For differentiation were propagated cells at 39° C in medium supplemented with 1% FCS with or without cytokines. At chosen time points cells were investigated for the expression of different markers by western blot and immunocytochemistry. As controls cells cultured at 33° C with 10% FCS for 3 days were used. We have shown that serum reduction alone is not sufficient for CSM14.1-cells to stop proliferating and begin differentiation. Following serum reduction and elevation of the temperature cells changed their morphology began to express specific band of the neuronal marker NeuN. Following cytokines treatment the mean length of cellular processes increased from 319 to 385 µm per cell, whereas the expression of neuronal markers such as NeuN and TH was not markedly changed. In conclusion, the differentiation cocktail consisting of interleukin 1(Il-1), Il-11, leukaemia inhibitory factor (LIF) and GDNF, does influence the outgrowth of neuritis but does not change the expression of mature neuronal markers at the protein level in CSM14.1 cells.

Key words: Cell transplantation, dopamine, immortalization, Parkinson's disease, predifferentiation

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative condition characterized by the progressive degeneration of dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SN) that innervate the striatum.

Experimental transplantation studies for PD have been carried out during the last 30 years on rodents and non-human primates using a wide spectrum of cells including chromaffin cells (9), human neuroblastoma cells (16), human retinal pigment epithelial cells (hRPE cells) (26), human amnionepithelial cells (14). More promissing results, however, have been obtained only after transplantation of DAergic tissue derived from ventral mesencephalon (VM) of different species into the striatum (13). Midbrain DAergic neurons are generated from precursor cells in the ventricular zone (VZ) of the developing VM. After mitosis the precursor cells migrate and start to express specific markers including tyrosine hydroxylase (TH) and differentiate into mature DAergic neurons. Immature DAergic neurons originating from aborted human embryos and implanted into the striatum of PD patients, restored DAergic neurotransmission and motor function (3). Limited donor tissue supply and

Abbreviations: BSA, bovine serum albumin; **CPu,** caudate putamen; **DA,** dopamine; **DAergic,** dopaminergic; **DMEM,** Dulbecco's modified Eagle's medium; **FCS,** fetal calf serum; **FGF,** fibroblast growth factor; **GDNF,** glial cell line-derived neurotrophic factor; **GFAP,** glial fibrilary acidic protein; **hRPE,** human retinal pigment epithelial cells; **ir,** immunoreactive, immunoreactivity; **IL,** Interleukin; **L-dopa,** L-3,4-dioxyphenylalanine; **LIF,** leukaemia inhibitory factor; **MAP,** microtubule associated protein ; **NeuN,** neuronal nuclei ; **NPCs,** neural progenitor cells; **NSE,** neuron specific enolase; **Nurr1,** nuclear receptor related protein 1; **OD,** optical density; **PBS,** phosphate saline buffer; **PD,** Parkinson's disease; **SN,** substantia nigra pars compacta; **SV,** Simian virus; **TH,** tyrosine hydroxylase; **VM,** ventral mesencephalon; **VZ,** ventricular zone.

ethical considerations hampered the development of this mode of therapy. Methods to grow neural progenitor cells (NPCs) *in vitro* have been developed based on immortalization (6, 25) or addition of growth factors to the culture media (21, 22). The basic issue of this study is the investigation of *in vitro* properties of the immortalized cell line CSM14.1, which was derived from a 14-day-old rat embryo and immortalized with the temperature – sensitive large T-antigen of Simian virus (SV) 40 (28). These cells have been shown to differentiate *in vitro* into tyrosine hydroxylase (TH)-expressing neurons with mature neuronal morphology (11).
Earlier it has been reported that transplantation of CSM14.1-cells into the adult rat caudate putamen (CPu) did not result in a DAergic differentiation (1,2). Nevertheless, when transplanted into CPu of neonatal parkinsonian rat, CSM14.1-cells differentiate into TH-containing neurons, improving motor behaviour (12).

Recently, many authors have focused their attention on the question whether pre-differentiation of cells *in vitro* can improve the therapeutical outcome and should be a key step before transplantation (4).

It has been shown that pluripotent lineage-restricted precursors derived from rat mesencephalic tissue could be expanded *in vitro* and differentiated into DAergic neurons using a combination of cytokines, growth factors, membrane fragments and striatal culture conditioned media (15, 20).

We were interested in finding out whether the *in vitro* differentiation of CSM14.1-cells could be influenced/improved after treatment with a differentiating medium including hematopoietic cytokines such as Interleukin 1(Il-1), Il-11, leukaemia inhibitory factor (LIF) and GDNF, because this combination has been shown to induce DAergic differentiation of a clonal line of mesencephalic NPCs (5).

MATERIALS AND METHODS

Cell Culture

Conditionally immortalized CSM14.1-cells were cultured and expanded in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 10 U/ml Penicillin, 100 µg/ml Streptomycin (Gibco) in a humidified incubator (95% air/5% CO_2, at 33° C). The cells were passaged every third day. Thereafter cells were cultured for up to 14 days in different culture conditions: permissive (33° C) or not permissive (39° C) temperature, normal (10%) or reduced (1%) content of FCS, with or without cytokines. The chosen cytokines cocktail (Carvey et al., 2001) contained: 100 pg/ml IL1, 1 ng/ml IL11, 1 ng/ml LIF and 10 ng/ml GDNF, solved in culture medium. All reagents were obtained from Gibco Life technologies (Karlsruhe, Germany).

Immunocytochemistry

For immunohistochemistry, cells were cultured overnight in a humidified incubator (95% air/5% CO_2, at 33° C) on poly L-lysine coated culture slides. CSM14.1-cells were washed with 0.9% sodium chloride and fixed 4% formaldehyde. After 3 rinses in 0.1M Tris-buffer (pH 7.4) cells were pre-incubated for 1 h in a blocking solution containing Triton-X100 (Sigma-Aldrich, Hamburg, Germany), 3% bovine serum albumin (BSA) (Sigma-Aldrich) and 2% normal goat serum (Gibco, Karlsruhe, Germany). Thereafter cells were incubated over night at 4° C with following primary antibodies diluted in the same blocking solution: mouse (ms) anti-Nestin (1:500, monoclonal, Becton-Dickinson, Heidelberg, Germany), ms anti- glial fibrilary acidic protein (GFAP) (1:400, monoclonal, Sigma-Aldrich), ms anti- microtubule associated protein (MAP5) (1:500, monoclonal, Sigma-Aldrich), ms anti-MAP2 (1:500, monoclonal, Sigma-Aldrich), ms anti-neuronal nuclei (NeuN) (1:1000, monoclonal, Chemicon, Hofheim, Germany), rabbit (rb) anti-neuron specific enolase (NSE) (1:250, polyclonal, Chemicon), ms anti- nuclear receptor related protein 1, Nurr1, (1:1000, monoclonal, Becton-Dickinson). Goat-derived anti-ms Cy3 and goat-derived anti-rb Cy3 were used as secondary antibodies.

For cresyl violet staining cells were fixed, washed in water and stained for 3 min with 0.1% cresyl violet. After washing in water they were mounted using DePeX (Serva).
Cell cultures were analyzed using a microscope (Leitz Aristoplan, Wetzlar, Germany) with respective filter units. Relative intensity of immunostaining was judged by the observer and scaled as - (not detectable), or if positive as + (sparse or weak), ++ (moderate) or +++ (extensive or strong).

Western blotting

For the western blot analysis CSM14.1-cells were passaged and cultured overnight in a humidified incubator (95% air/5% CO_2, at 33° C) in Petri dishes.
Thereafter the cells were rinsed with 0.1 M phosphate saline buffer (PBS) (pH 7.4), trypsinized (Gibco Life Technologies), centrifuged (10 min at 400 *g*), washed and neutralized with DMEM containing 10% FCS. After a final centrifugation (10 min at 400 *g*), cells were resuspended in PBS. The viability and the number of cells were determined by trypan blue exclusion (Sigma-Aldrich). The cells were then lysed by several freeze/thaw cycles. Equal total cell protein concentrations were determined using a spectrophotometer (Model DU640, Beckman, Fullerton, CA, USA) and a bicinchoninic acid assay (Pierce Chemical Co, Rockford, IL, USA). Comparable concentrations of whole protein lysates were boiled for 5 min in SDS (sodium dodecyl sulphate) sample buffer. Twenty micrograms of protein dissolved in 20 µL SDS sample buffer was loaded in each lane.

After electrophoresis, proteins were blotted onto nitrocellulose membranes (Amersham Pharmacia Biotech, Freiburg, Germany). The membranes were washed in PBS and blocked 1 h at room temperature in solution, containing 0.1M PBS (pH 7.4) 0.1% Tween 20 (PBS-T) and 1% BSA. Thereafter membranes were incubated with primary antibodies diluted in a blocking solution, overnight, at 4° C. The following primary antibodies were used: ms anti-ß-actin as an internal control, (1:3000, monoclonal, Sigma), rb anti-

NSE (1:1000, polyclonal, Chemicon), ms anti-NeuN (1:5000, monoclonal, Chemicon), ms anti-TH (1:3000, monoclonal, Sigma-Aldrich), ms anti-Nurr1 (1:1000, monoclonal, Becton-Dikinson), ms anti-GFAP (1:4000, monoclonal, Sigma-Aldrich). Membranes were washed in PBS-T (4 × 15 min) and incubated for 1 h at room temperature with secondary antibodies conjugated with horseradish peroxidase (anti-mouse, 1 : 5000 or anti-rabbit, 1 : 10 000, both Vector Laboratories, Burlingame, CA, USA). After washing the membranes in PBS-T, the peroxidase activity was visualized with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech).
To avoid crossover reactions we performed two Western blots to detect the target protein and the internal contron (ß-actin) separately.

Measurements and statistics

Eight independent probes of one experimental condition of cultured cells were stereologically evaluated: The projection area (40x), the number of cells (10x) and the length of processes (10x) were estimated by means of point counting (10). For this purpose, a light microscope equipped with a projection mirror was used to project appropriate counting grids in the visual field. The Western blots were digitized using a high resolution transparent flat bed scanner (600 dpi, 8 bit). Images were corrected with regard to background and evaluated densitometrically (19) after interactive designation of spots. 5 blots for one experimental condition were quantified to obtain a mean densitometric value shown as gray values between 0 and 255 in diagrams. Differences of the stereological results were tested applying the Mann-Whitney test of SPSS 11.0.

RESULTS

Cell proliferation

The proliferation of CSM14.1-cells was assessed in culture conditions of 33° C and 10% of FCS. Cell proliferation in culture during 3, 7 and 10 days indicate a strong increase of the absolute cell number from 12.3 millions at 3 day to 18.3 millions at day 10. Thereafter, their number at 14 days decreased to 9.9 millions (Fig. 1A). The number of CSM14.1-cells per mm^2 cultivated with or without cytokines at 39° C with 1% FCS was counted after 14 days of cultivation (Fig. 1B). The number of cells was 129.2 ± 7.83 cells per mm^2 and 202.1 ± 6.04 in cultures with and without cytokines, respectively.

Cell morphology

Cells propagated at 33° C with 10% of FCS for 3 days were designated as controls. No changes were found between control cells and cells with only 1% of FCS (Fig. 2A, B). The undifferentiated cells with small bodies had short processes and an epithelial-cell-like phenotype. In contrast, cells supplemented with 1% FCS and cultured at nonpermissive temperature, after 14 days assumed larger somatas and longer processes and began forming a connective network (Fig. 2C).

In presence of cytokines at 39° C with 1% FCS CSM14.1-cells extended longer processes as compared to those without cytokines (Fig 2D, C). For the cells without cytokines the mean length of outgrowths was 319.1 ± 21.05, and for those with cytokines 385.1 ± 10.02 (µm per cell) (Fig. 2E), whereas the mean area of somatas was not markedly changed (Fig. 2F). The quantification was performed on cresyl violet-stained cell cultures.

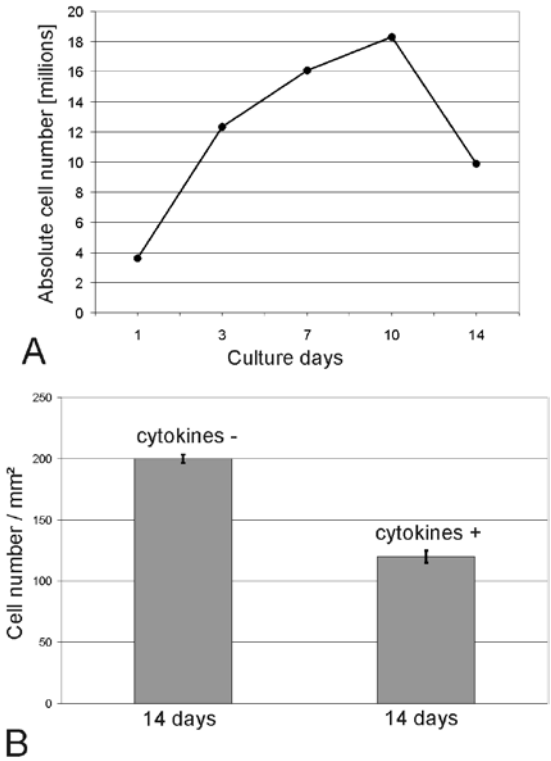

Figure 1. Proliferation of CSM14.1-cells. (A) - Absolute number of cells in three Petri dishes. The cells proliferate at 33° C in medium supplemented with 10% FCS, increasing the cell number from 1,5 million at day 0 to 16,1, 18,3 million at days 3 and 10 respectively. Thereafter cell number is decreasing, reaching 9,9 million cells at day 14.
(B) - Cell number per mm² in cultures of CSM14.1-cells propagated at 39° C and 1% FCS for 14 days with or without cytokines. In cultures without cytokines cell number per mm² was greater (202.1 ± 6.04) than in cultures without cytokines (129.2 ± 7.83) (* p=0.001).

Protein Expression

Nestin

Nestin, a class IV intermediate filament protein, which is expressed in developing neural stem and progenitor cells, has been used to describe the "stemness" of cells in different culture conditions. At 33° C with 10% FCS cells were highly immunoreactive (ir) for progenitor cells marker nestin (Fig. 3A). After serum

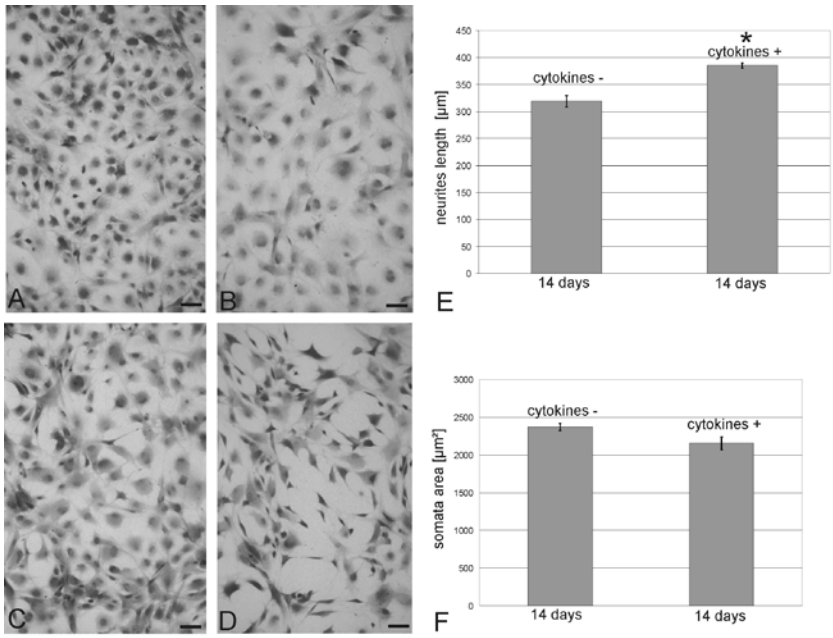

Figure 2. Morphology of cresyl violet-stained CSM14.1-cells under different culture conditions. There are no morphological changes between control cells (A) (33° C + 10% FCS for 3 days) and cells cultured for 14 days in serum reduced medium (B). Cells cultured for 14 days in differentiating conditions (39° C + 1% FCS), in contrast resemble multipolar neuronal morphology with sprouting outgrowths (C). After cytokine expansion for 14 days (39° C + 1% FCS) cells (D) have also multipolar somata and assume longer processes. The mean neuritis lengths differ between cultures propagated without (319.1 ± 21) or with cytokines 385.1 ± 10.02 (E) (*$p \leq 0.05$) µm per cell. The mean somata areas (F) do not differ between cultures with or without cytokines ($p \geq 0.05$). Scale bar= 35 µm

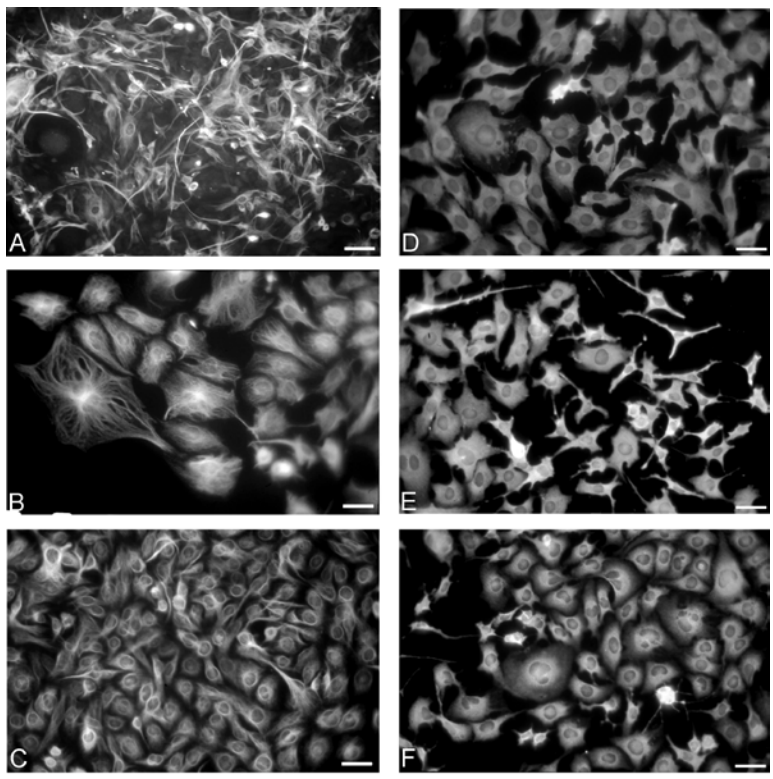

Figure 3. (A-C) - Nestin expression of CSM14.1-cells in different conditions. Nestin is expressed in control cells (A), and no changes in the intensity are detectable following serum reduction after 7 (B) and 7 (C) days. Neuronal marker MAP5 (microtubule associated protein) is also expressed in controls (D), and serum redcuction have no influence on staining intensity after 7 (E) or 14 (F) days of culturing. Scale bar=25 µm in B, 35 µm in A, C – F.

reduction no decrease in nestin expression was detected after 7 (Fig. 3B) and 14 days (Fig. 3C) of culturing.

MAP5. MAP5 is expressed already in control cultures (Fig. 3D). No changes in the MAP5-expression were observed after serum reduction at 7 (Fig. 3E) and 14 (Fig. 3F) days.

MAP2

Was not detectable by immunohisto-chemistry or western blotting neither in control culture nor after serum reduction. MAP2 was even not detected after temperature elevation and addition of cytokines.

NSE

In western blots NSE immunoreactivity (ir) was very weak in control CSM14.1-cells (Fig. 4A). After serum reduction its expression remained unchanged until 14 days (Fig. 4A). Immunocytochemistry revealed no significant changes in NSE-staining intensity after 14 days of serum reduction (Fig. 4F) when compared to controls (Fig. 4E). After serum reduction and shifting of the temperature no changes in its expression were detected (Fig. 4B). An increased expression of NSE expression was detected after 3 days in culture with cytokines (Fig. 4B). This was down-regulated at 7 days, but remains detectable until 14 days (Fig. 4B).
The membranes were re-probed using ß-actin (42 kDa). Similar amounts of the housekeeping protein ß-actin were found in all lanes (Fig. 4C, D).

NeuN

The band in 48 kDa, specific for immature neurons was detected already in control cultures (Fig. 5A). After serum reduction its expression was up-regulated and remained constant during 14 days (Fig. 5A). In protein lysates of cells cultured at 39° C with 1% FCS the specific band in 46 kDa became obvious: its expression remained constant during further 14 days (Fig. 5B). Cells, cultured in the presence of cytokines, also expressed the specific band of NeuN (Fig. 5B).

Nurr1

This protein was clearly detectable in control cultures with a band of 72 kDa (Fig. 6B). The comparison of OD-s of the lane from control cell lysates and from cells after serum reduction revealed no significant alterations, indicating comparable means in control cells (200.71 ± 11.15) and cells from culture day 3 (202.8 ± 3.15), 7 (201.1 ± 10.37), 10 (210.0 ± 9.10) and 14 (207.3 ± 7.10) (Fig. 6A). Immunocytochemistry revealed no ir for Nurr1 either in control culture or in cells cultivated with 1% of FCS. At 39° C with 1% FCS the expression was up-regulated after 3 days and gradually increased during 14 days in culture (Fig. 6B). The comparison of mean OD-s of Nurr1 bands from the same culture days of cytokine-treated and untreated cultures revealed significant difference only after 14 days, whereby the mean value of untreated culture was greater (Fig. 6C) (*$p \leq 0.05$). As shown in Fig 6C both cell cultures (with or without cytokines at 39° C) indicate increased Nurr1 expression compared to controls (***$p \leq 0.001$).

TH

TH was weakly detected in control cultures with a band of 68 kDa (Fig. 6E). After serum reduction its expression was up-regulated - compared to the control (96.6 ± 6.50) the mean OD showed a gradual increase reaching 115.3 ± 6.36 at 3, 121.7 ± 8.05 at 7 and 130.9 ± 12.67 at 10 days. After 14 days expression was down-regulated, showing a mean OD 103.7 ± 11.30 (Fig. 6D). After temperature elevation the expression of TH was up-regulated compared to control and remained constant until 14 days (Fig. 6E). Cytokines-treated cultures compared with untreated showed no significant changes in TH expression to any time point ($p \geq 0.05$) (Fig. 6F). Compared to the controls, CSM14.1-cells propagated at 39° C with or without cytokines revealed a significant increase in TH expression (***$p \leq 0.001$) (Fig. 6F).

GFAP

No ir for GFAP at any time point was found.

DISCUSSION

CSM14.1-cells (7) were derived form the VM of E14 rats and immortalized via transfection with a retroviral vector coding for temperature sensitive large T-antigen. We showed that at 33° C in presence of 10% serum, cells multiplied their number until 10 days of culture. Cells in these conditions were ir for nestin- a marker for precursor cells in the CNS (8). Neither nestin expression nor cell morphology was altered after serum reduction to 1%. Haas and Wree (11) have shown that serum reduction paired with temperature elevation provoked a decrease in nestin expression and

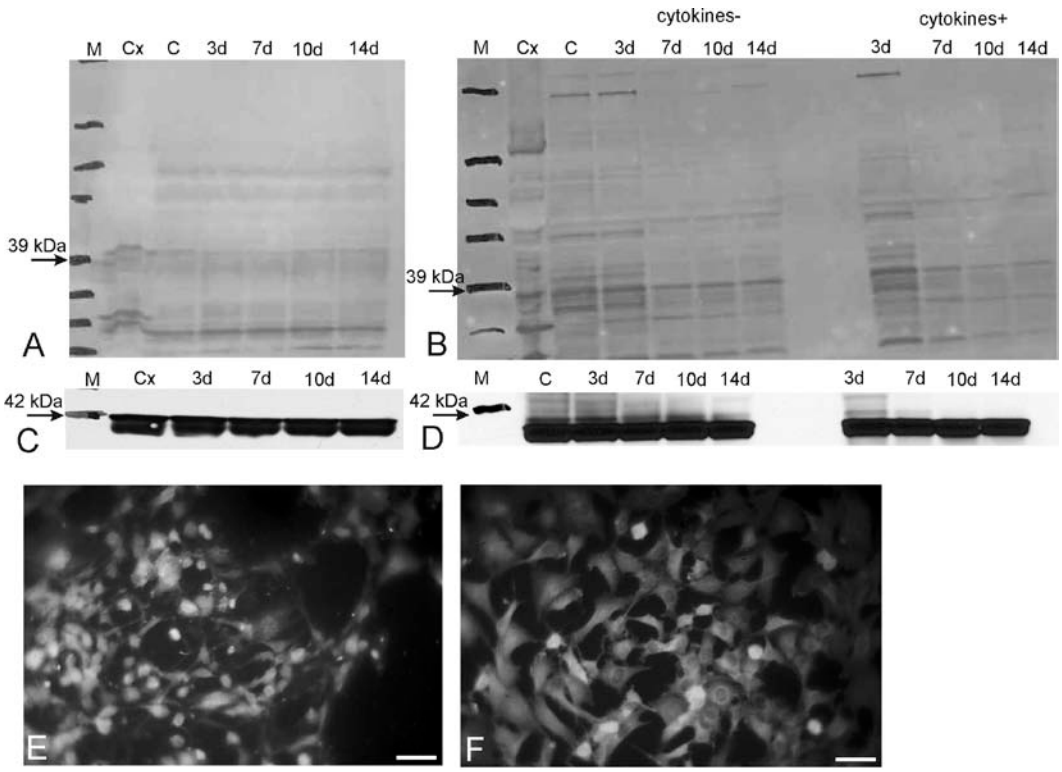

Figure 4. Western blots (A, B) and immunocytochemical staining for NSE (E, F). NSE is detected in control cultures by western blotting (A) and immunocytochemistry (E). The expression is not altered following further culturing for up to 14 days in serum reduced medium at 33° C as shown by western blot (A). Consistently, no changes are detected by immunocytochemistry between controls (E) and after serum reduction at day 14 at 33° C (F). Western blot of cells at 39° C + 1% FCS shows no changes compared to controls (B). In cultures with cytokines, NSE expression is slightly upregulated at day 3, thereafter get down, remaining detectable up to 14 days. (C) and (D) are presenting the housekeeping marker ß-actin that shows equal quantaties in each lane. (B). M: protein marker, C: CSM 14.1 cells control cultures (33° C with 1% FCS), Cx: cortex. Scale bar=35 µm in E and F.

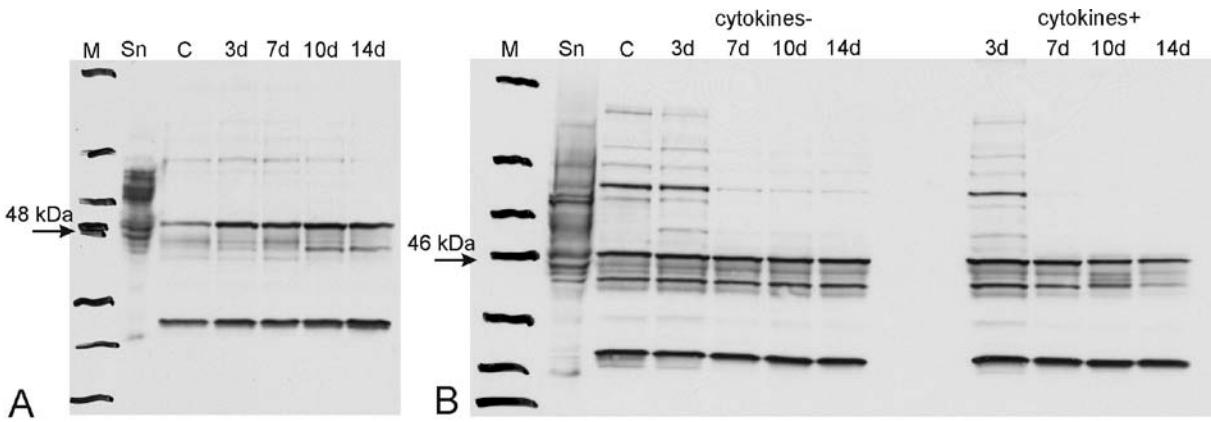

Figure 5. (A) - Western blots showing the unspecific band (48 kDa) of NeuN (neuronal nuclei) in control. After serum reduction its expression is up-regulated at 3 days, and is constant for up to 14 days. (B) - after serum reduction and temperature shifting appears the specific band of 46 kDa, and no changes are revealed between cultures with or without cytokines. M: protein marker, Sn: Substantia nigra, C: CSM 14.1 cells control cultures (33° C with 1% FCS)

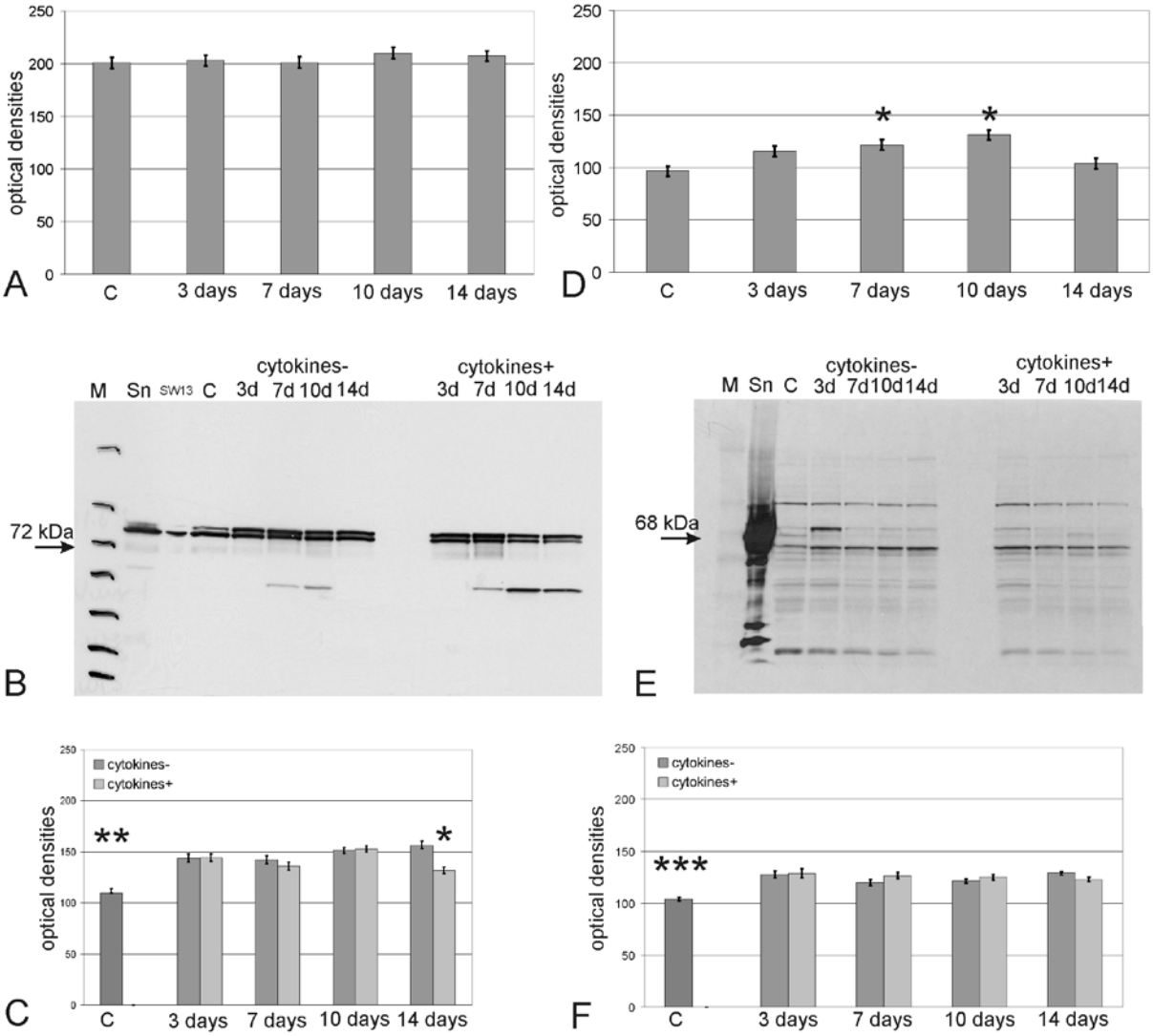

Figure 6. (A): The comparison of mean ODs of Nurr1 (nuclear receptor related protein 1) bands from control cultures and those after serum reduction indicates no significant changes. Western blot analysis of cell lysates from cultures after serum reduction and expansion at 39° C with or without cytokines (B). Both cultures at different time points compared to control show an up-regulation of Nurr1 expression p*≤0.001, and no significant changes in its expression following cytokines treatment, which is clearly shown by measerument of bands ODs (C).
TH is detected with a band of 68 kDa (E) and is up-regulated after serum reduction as shown densitometry (D). Densitometry (F) reveals a significant increase in TH-expression between controls cultures and cultures from differentiating conditions (39° C + 1% FCS) (p*≤0,001), whereas no changes are detected between cultures with or without cytokines. M: protein marker, C: control, Sn: Substantia nigra, SW13: lysat of SW13 cells, recommended as positive control by manufacturer

changes in cell morphology. We suggest that serum reduction alone is not sufficient to make the cells to stop proliferation and undergo terminal differentiation. When cultured at 39° C with 1% FCS, cells show a clearly detectable specific band of NeuN in 46 kDa (typical to mature neurons) in western blot and an increase in Nurr1 expression compared to control (33° C with 10% FCS), which is in a good agreement with data obtained by Haas and Wree (11).

In the present study MAP2 has not been detected at any time point. In contrast, Vernon and Griffin (27) have shown that CSM14.1-cells differentiate in non-permissive conditions (39° C and 1% FBS), and express MAP2. However, MAP2 in their experiments became clearly detectable only after 3 weeks of culturing. It is known that MAP2 antigen concentration varies with the degree of dendritic differentiation, and that its expression is proportional to the state of differentiation (23). It is notable, that after serum reduction CSM14.1 cells in the present study expressed NeuN, which occurs in association with terminal differentiation (18). NeuN is a

nuclear marker, whereas MAP2 antibody predominantly stained the dendritic processes. It can be proposed that CSM14.1-cells need longer time in culture to assume functionally mature outgrowths.

In concordance with data from Haas and Wree (11), GFAP expression was not observed at any time point. In both cases monoclonal antibodies against GFAP have been used. These results are contrary to those obtained by Vernon and Griffin (27). Using polyclonal antibody against GFAP they have shown that GFAP was expressing in undifferentiated CSM14.1-cells, and reduced with differentiation.

The temperature-sensitive variant of SV40 large T-antigen has been the main choice for the conditional immortalization. However, many studies failed to demonstrate full differentiation of the immortalized cells by only switching the temperature from the permissive to the nonpermissive one (17). It can be concluded, that additionally factors like growth factors, cytokines and appropriate substrates are needed in addition to elevation the temperature to provoke complete differentiation of immortalized cells. Whereas earlier studies have examined CSM14.1-cells in culture (12) and following their transplantation *in vivo* (12), this is the first study to investigate the differentiation of these cells in the presence of cytokines.

We used the combination of IL1, IL11, LIF and GDNF. This combination of haematopoietic cytokines has been demonstrated to participate in the induction of DAergic phenotype and cellular maturation of mesencephalic progenitor cells (5, 15, 20). In our study cell cultures propagated with or without cytokines at 39° C (1% FCS) were compared in the line of their morphology. The morphological differences between two cultures became most obvious after 14 days. The cells of both groups contained somata of comparable sizes, whereas cells with cytokines assumed longer processes. This is in accordance with studies where cytokines caused maturation of cells and commitment to the neuronal phenotype (5, 24). The number of cells expanded with or without cytokines differed significantly after 14 days of culturing, showing larger number of cells in the culture without cytokines.

Despite these facts we found little evidence for further differentiation of CSM14.1-cells under these differentiating conditions, as expression of differentiation marker proteins following cytokines addition was apparently unchanged. To our knowledge, the expression of neuronal markers typical to mature neurons, is a more reliable indicator of neuronal differentiation, than cell morphology, and we are inclined to believe, that in spite of morphological changes, the differentiation of CSM14.1-cells is not significantly altered by cytokines addition.

The same combination of haematopoietic cytokines have been already claimed to induce DAergic differentiation in clonal-expanded mesencephalic progenitor cell line MPC-C9[19]. Concerning influence of used combination of cytokines on CSM14.1-cells, we are cautious in comparing our results with those obtained by Carvey et al (5). The cells used by Carvey et al (5) were expanded as “proliferating spheres” in the presence of growth factors making them in principle incomparable with immortalized cells used in our experiment, whereas both cell lines derived from rat VM.

In conclusion, CSM14.1-cells proliferate at permissive temperature and begin to differentiate after serum reduction and temperature elevation to the non permissive. CSM14.1-cells differentiated in culture for up to 14 days independently from cytokines addition. It can be only suggested, that either CSM14.1-cells need longer time to respond to this combination of cytokines or, additional factors are to be identified as being required for enhancement of DAergic differentiation in this cell line.

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