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Effects of FGF-2 and EGF removal on the differentiation of mouse neural precursor cells

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ABSTRACT

Cell therapy for neurological disorders has advanced, and neural precursor cells (NPC) may become the ideal candidates for neural transplantation in a wide range of diseases. However, additional work has to be done to determine either the ideal culture environment for NPC expansion *in vitro*, without altering their plasticity, or the FGF-2 and EGF mechanisms of cell signaling in neurospheres growth, survival and differentiation. In this work we evaluated mouse neurospheres cultured with and without FGF-2 and EGF containing medium and showed that those growth factors are responsible for NPC proliferation. It is also demonstrated that endogenous production of growth factors shifts from FGF-2 to IGF-1/PDGFb upon EGF and FGF-2 withdrawal. Mouse NPC cultured in suspension showed different patterns of neuronal localization (core *versus* shell) for both EGF and FGF-2 withdrawal and control groups. Taken together, these results show that EGF and FGF-2 removal play an important role in NPC differentiation and may contribute to a better understanding of mechanisms of NPC differentiation. Our findings suggest that depriving NPC of growth factors prior to grafting might enhance their chance to effectively integrate into the host.

Key words: neural precursor cells, neurosphere, fibroblast growth factor 2, epidermal growth factor, differentiation

INTRODUCTION

Evidence of neurogenesis in the adult brain of birds (Goldman and Nottebohm 1983), rodents and primates (Kuhn et al. 1996, Gould et al. 1999, Kuhn and Svendsen 1999), and the demonstration of stem cells in specific brain regions, such as the subventricular zone and the hippocampus (Eriksson et al. 1998, Van Praag et al. 2002), brought new perspectives for cell therapy and neural regeneration (Svendsen and Smith 1999). Al-

though these cells in the adult brain have decreased plasticity and are not as easy to manipulate and to grow in culture as their embryonic stem cell counterparts (Lewin 1998, Thomson et al. 1998), they do not involve ethical questions.

During the development of the central nervous system, there is extensive proliferation of neuroepithelial cells lining the ventricular walls which give rise to neurons, astrocytes and oligodendrocytes of the brain (Jacobson 1991). An experimental model to study neural stem cells is the heterogeneous free floating



sphere is derived from a single stem cell that, by asymmetrical division, gives rise to another stem cell and one progenitor cell. The progenitor cells, in turn, give rise only to other progenitor cells. In this way, only a small fraction of the neurosphere corresponds to real stem cells (Reynolds et al. 1992). Here we use the terminology neural precursor cells (NPC) to describe both cell types within the neurosphere (Svendsen et al. 1999, Svendsen and Caldwell 2000).

Cell therapy for neurological disorders has advanced, and NPC may become the ideal candidates for neural transplantation in a wide range of diseases such as Parkinson (Dunnett and Bjorklund 1999). Previous studies have shown that mouse NPC can be pluripotent when exposed to the correct environment and produce hematopoietic cells in irradiated mice (Bjornson et al. 1999) and cells from distinct mesodermic lineages when injected in mouse blastocysts or chicken embryos (Clarke et al. 2000). Neurospheres are usually cultured in fibroblast growth factor 2 (FGF-2) and epidermal growth factor (EGF) containing medium in the first weeks and switched to EGF alone after 4 weeks. It was also shown that the acquisition of EGF responsiveness by neural precursor cells is promoted by FGF-2 in the early development *in vitro*. After several cell divisions, the same cell type may respond to both EGF and FGF-2 (Ciccolini and Svendsen 1998). Yet, FGF-2 and EGF mechanisms of cell signaling in neurospheres growth, survival and differentiation are still unclear. It was previously reported that a method of neurospheres passaging, that maintain cell-cell contact, was also important for neurosphere growth (Svendsen et al. 1998).

Manipulation of environmental signals can make the cells differentiate in specific cell types and may help us to understand the mechanisms of neural and glial development to be assessed. Despite the advances in stem cell studies, additional work has to be done to determine the ideal culture medium for NPC expansion *in vitro* without altering their plasticity. It is likely that on and off periods of growth factors might provide specific patterns of cell proliferation, migration and differentiation. In this work we evaluate mouse neurospheres cultured

MATERIALS AND METHODS

ETHICAL ISSUES

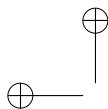
This work was developed under the approval of the Ethics Committee of Universidade Federal de São Paulo (UNIFESP), file 0976-04.

HARVESTING AND CULTURING MOUSE NPC

mNPC were obtained from E14 (embryonic day 14) C57BL/6 mouse embryos. The fetuses were placed in a Petri dish containing PBS (phosphate buffered saline)/2% glucose, and the dissection was made under magnifying lens. The brains were sectioned and the tissue was incubated with Trypsin-EDTA solution (Invitrogen) for 15min at 37°C. Trypsin was inactivated with fetal bovine serum, and, after cell sedimentation, the supernatant was removed and the cells were dissociated. Cell suspension was counted in a hemocytometer and the cells were seeded in 10 mL in a T25 flask at a density equivalent to 100,000 cells/mL. Each flask contains cells derived from a pool of 3 fetuses and all experiments were performed using three independent cultures of mNPC. The culture media is composed by 70% DMEM (Dulbecco's modified eagle medium; Invitrogen), 30% F12 (Invitrogen), 1% PSA (penicillin-streptomycin-amphotericin, Invitrogen), 2% B27 (Invitrogen), 20ng/mL EGF (Sigma), 20ng/mL FGF-2 (R&D), and 5µg/mL heparin (Sigma). Cells were maintained in an incubator at 37°C under a 5% CO₂ atmosphere. Every 3 days, half of the volume was replaced with fresh medium.

GROWTH FACTORS WITHDRAWAL

After neurosphere expansion (as described above), half of the population within one flask was kept in the complete media (control group – CTR) and the other half was cultured in the absence of growth factors (E/F less). The spheres were transferred to conical tubes and washed carefully 3 times with pre warmed DMEM. If necessary, the spheres were centrifuged at 130Xg. The spheres were put in growth factors free medium



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PREPARATION OF NEUROSHERES SLICES

Whole neurospheres from the CTR and E/F-less groups were taken from the flasks, washed with PBS to remove the excess of culture medium and fixed in 4% PFA (paraformaldehyde) for 1h at room temperature. Neurospheres were washed three times with PBS and then transferred to PBS/10% sucrose solution for 1h at 4°C, PBS/20% sucrose solution for 1h at 4°C and, finally, PBS/30% sucrose solution at 4°C overnight. Neurospheres were then mounted in Histo Prep (Fisher Scientific) and frozen in dry ice. Spheres were sliced at 10 μ m on a cryostat and placed on silanized slides (Superfrost slides, Fisher Scientific).

5-BROMO-2'DEOXYURIDINE (BrdU) INCORPORATION

BrdU (0.2 μ M) was added to the medium for 14h to the CTR and E/F less groups. To visually inspect the localization of proliferating cells in the neurosphere, whole neurospheres were sectioned in a cryostat and placed on silanized slides. For BrdU counting, spheres were dissociated using Trypsin and plated onto poly-lysine/laminin coated coverslips before immunocytochemistry. Sections or coverslips were incubated in HCl 1.5M for 30 min under gentle shaking, washed 3X 10 min in PBS and blocked in 5% normal goat serum and 0.1% TritonX-100 in PBS. The slides were incubated with anti-BrdU (Axyll/Accurate Chemical & Scientific Corporation, rat IgG, 1:200) for 2h and washed three times in PBS. Cells were incubated with the secondary antibody (Alexa 488 anti-rat IgG, Molecular Probes) for 1h and washed three times in PBS. DAPI solution (Sigma, 0.3 μ g/ml) was used as a nuclear stain. Cells were analyzed under a fluorescence microscope (Nikon, model Eclipse E600FM, Japan). The percentage of BrdU positive cells from the coverslips was statistically analyzed by using the ANOVA test with post hoc test Newman-Keuls with a significance level set at $p < 0.05$.

GROWTH CURVE

Isolated single neurospheres were placed in 96-multiwell plates to avoid neurospheres fusion. Multiwell

spheres were maintained in the presence (CTR) or the absence (E/F-less) of growth factors for 11 days. The diameter of the neurospheres were measured at 3 days. We used the diameter measures to calculate the volume of the spheres during 11 days. For both CTR and E/F-less groups, 4 distinct neurospheres were measured in each triplicate. Growth curve results were subjected to the Student t test with a significance level $p < 0.05$.

IMMUNOCYTOCHEMISTRY FOR β -TUBULIN III, GFAP (GLIAL FIBRILLARY ACIDIC PROTEIN) AND GAL-C (GALACTOCEREBROSIDE C)

The slides were blocked and permeabilized in 5% normal goat serum and 0.1% Triton X-100 in PBS for 30 min (except for Gal-C, in which detergent was not added). Primary antibodies for β -tubulin III (mouse IgG, 1:300), GFAP (DAKO, rabbit IgG, 1:100), Gal-C (Chemicon, mouse IgG3, 1:200) were added. Cells were incubated overnight at 4°C. The slides were washed in PBS and incubated with the secondary antibodies (Alexa 546 anti-mouse IgG, Alexa 488 anti-mouse IgG or Alexa 488 anti-rabbit IgG, Molecular Probes, 1:250). After washing with PBS, DAPI solution (Sigma, 0.3 μ g/ml) was used as a nuclear stain. Cells were analyzed under a fluorescence microscope. β -tubulin III was considered a reliable marker for neuronal differentiation because it was shown previously that, besides being expressed by both mature neurons and neural progenitors, β -tubulin III⁺ migrating cells in neurospheres adopt a neuronal phenotype and express neuronal markers (GABA, glutamate) and neuronal markers (Neu-N, MAP-2ab) (Ostenfeldt and Svendsen 2004). GFAP is used as an astrocytic marker but it can be also expressed by immature neural progenitor cells.

TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE-MEDIATED BIOTINYLATED UTP NICK END LABELING (TUNEL)

In situ cell death detection kit (Roche) labels apoptotic cells, based on labelling of DNA strand breaks.



blocked and permeabilized in 5% normal goat serum and 0.2% Triton X-100 in PBS for 15 min at room temperature. Cells were washed one time with PBS and the enzyme and nucleotide mix were added (Enzyme-TdT and Label-nucleotide) and incubated for 30 min at 37°C. Cells were washed 3 times with PBS and the nuclei were labelled with DAPI for 5 min. Cells were washed with PBS, mounted and analyzed under a fluorescence microscope. TUNEL data were analyzed using the Student *t* test (significance set at $p < 0.05$).

RNA EXTRACTION AND CDNA SYNTHESIS

Total RNA was isolated by using TRizol (Invitrogen) reagent according to manufacturers' protocol. Total RNA concentration and integrity were determined by spectrophotometer readings at absorbance 260nm and 280nm and by observation of the Ribosomal RNA bands in a 1% agarose gel electrophoresis respectively. To avoid DNA contamination, the RNA was previously treated for 30 min at 37°C with 1 U RQ1 RNase-Free DNase (Promega) in presence of 20 U RNaseOUT™ (Invitrogen) RNase Inhibitor and the DNase was inactivated by a 95°C incubation for 15 min and immediately chilled on ice. First strand cDNA synthesis was performed using SuperScript™ II Reverse Transcriptase (Invitrogen) as suggested by the manufacturer using 5μg of total RNA. Resultant cDNA was then used for PCR as described below.

QUANTITATIVE REAL-TIME PCR

Expression analysis of mRNA was performed in the ABI PRISM 7700 *sequence detection system* (Applied Biosystem, EUA) using SYBR®-Green amplification detection system. Each reaction was performed in a final volume of 20μL using cDNA reversed transcribed from 25ng of the RNA, 10μL of the SYBR®-Green Universal PCR Master Mix and 1μL of each forward and reverse primers (10μM each) shown below. We conducted the Real-Time PCR reactions separately using the following temperature protocol: 50°C-2 min, 95°C-10 min, and 50 cycles of 95°C-15s and 60°C-1 min, followed by a dissociation curve protocol to check the specificity

of all reactions was approximately 100% (> 95%), we were able to use $2^{-\Delta C_t}$ parameter to express relative expression data, taking TBP (TATA binding protein) as endogenous control. Real-Time PCR experiments were submitted to the Student *t* test with a significance level set at $p < 0.05$. Primers, forward and reverse, respectively, are represented in the 5' → 3' direction:

β-tubulin III: AGACCTACTGCATCGACAATGAAG
and GCTCATGGTAGCAGACACAAGG;

GFAP: AAGAGTGGTATCGGTCCAAGTTTG
and CAGTTGGCGGCGATAGTCAT;

EGF: CCAAACGCCGAAGACTTATCC
and CTTATTACCGATGGGATAGCCC;

FGF2: CCAACCGGTACCTTGCTATGA
and TTCGTTTCAGTGCCACATACCA;

IGF1: GCCACACTGACATGCCCAAG
and TGCACTTCCTCTACTTGTGTTCTTC;

NT3: TTACAGGTGAACAAGGTGATGTCC
and CCGGCAAACCTCTTTGATCC;

PDGFa: CATTCGCAGGAAGAGAAGTATTG
and CTGGTCTTGCAAACCTGCGGG;

PDGFb: GAAAGCTCATCTCGAGGGAGG
and GCGTCTTGCACTCGGCG;

Nestin: TGACCATTTAGATGCTCCCCAG
and GTCCATTCTCCATTTCCCATTC;

TBP: GAATCTTGGCTGTAACTTGACCT
and TCTTATTCTCATGATGACTGCAGCA.

RESULTS

To assess if there was a significant difference in growth and in the expression of growth factors and neural specific proteins between the CTR and E/F-less groups, three independent cultures of mNPC derived from E14 embryos were subjected to growth factors deprivation for 11 days, followed by analyses of proliferation, cell death and differentiation.

To confirm if removal of EGF and FGF-2 would have an effect in the proliferation rates of neurospheres in suspension. BrdU incorporation and growth rates of



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size increment for the E/F-less group, as observed in the growth curves (Fig. 2). However, the percentage of TUNEL positive cells did not change significantly during the growth factors starvation process, when compared to the CTR group (Fig. 3).

Given that growth factors removal leads to a decrease in cell proliferation, we expected to find cells undergoing differentiation, even in suspension. Our results show that staining of mNPC for neuronal and astrocytic markers (β -tubulin III and GFAP respectively) yielded a different distribution and localization of neurons in the E/F-less and CTR groups. GFAP was found across the whole neurosphere whereas β -tubulin III positive cells were concentrated in the neurosphere core (Fig. 4). In order to check if there is an increase in the transcripts of β -tubulin III, *gfap* and *nestin*, we performed quantitative PCR. The results showed a decreased expression of *nestin* and *gfap*, but a tendency of increase in β -tubulin III expression in the E/F-less group when compared to CTR (Fig. 5).

Our results showed important differences in the proliferation and distribution of cell populations within the neurosphere after growth factors withdrawal. Then, we asked if growth factors produced by the NPC could be differentially expressed and play a role in cell survival and differentiation after EGF and FGF-2 removal. The expression of a number of growth factors that are relevant for cell proliferation and survival (*egf* – Epidermal Growth Factor, *fgf-2* – Fibroblast Growth Factor 2, *igf-1* – Insulin-like Growth Factor 1, *nt3* – Neurotrophin 3, *pdgfa* and *pdgfb* – Platelet Derived Growth Factor α and β) was measured in the CTR and the E/F-less groups by using the Real-Time PCR technique. Our Real-Time PCR results showed that, after growth factors withdrawal, mNPC decreased the expression of *fgf-2* and increased the expression of *igf-1* and *pdgfb* (Fig. 5).

DISCUSSION

This work demonstrates that mNPC can survive and differentiate in suspension after EGF and FGF-2 removal, but have reduced its proliferation capability. Only very few BrdU positive cells were found in the E/F-less

surement of growth rates also showed that neurospheres subjected to growth factor removal did not grow during the period of growth factor absence (Fig. 2). However, cell death percentage is around 5% in both CTR and E/F-less groups, indicating that cells can survive under this condition. The same cell survival under growth factor deprivation must be due to the differential expression of growth factors and cytokines in the neurosphere. Immunocytochemistry assays showed that mNPC cultured in suspension differentiated into the three basic cell types: neurons, astrocytes and a few oligodendrocytes (data not shown), in both the E/F-less and CTR groups. This reproduces *in vitro* the development of the cerebral cortex *in vivo* (Wright et al. 2006).

Campos and colleagues (2004) have recently proposed a three-dimensional model of mouse neurosphere based on the expression of specific proteins and cell labeling. This model shows the presence of immature (Nestin⁺) and cycling (BrdU⁺) cells in the outer layer of the neurosphere, as well as laminin $\alpha 2$ and integrin $\alpha 5$. Yet, the astrocytic marker GFAP is expressed across the whole neurosphere, while some extracellular matrix proteins and the neuronal marker β -tubulin III are preferentially expressed in the neurosphere core. Our findings are in agreement with the mouse model for GFAP and β -tubulin III of mNPC cultured in suspension in the presence of EGF and FGF-2 (CTR group) (Fig. 4). We hypothesize that this distribution of neurons is caused by a decreasing gradient of growth factors from the outer layer towards the center of neurospheres. Since the concentration of EGF and FGF-2 inside the sphere might be lower than outside in the CTR condition, cells in the neurosphere core are able to stop proliferation and start differentiation even in suspension.

On the other hand, in the E/F-less group, in the absence of growth factors in the culture medium, mNPC are concentrated on the border of the neurospheres, where GFAP positive cells had the same localization (Fig. 4). We hypothesize that, in the CTR group, cells from the neurosphere core are exposed to a lower concentration of growth factors than are the cells from the border due to the gradient. This condition induces

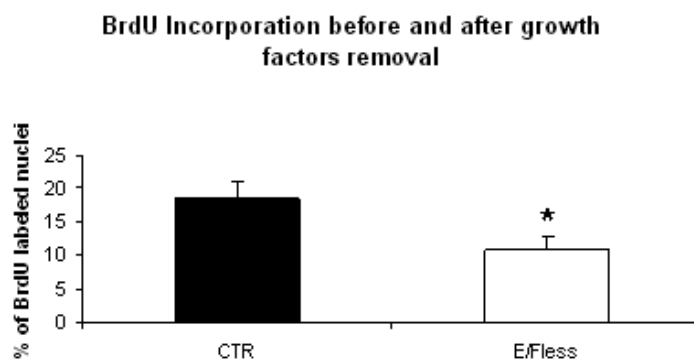


Fig. 1 – Proliferation analysis of mNPC by BrdU incorporation after growth factor removal. The differences in the percentage of BrdU positive cells between the CTR and the E/F-less groups after 11 days of growth factor withdrawal were statistically significant ($n = 3$; $P < 0.05$). Abbreviations: 11d – duration of treatment; CTR – neurospheres submitted to regular conditions; E/F-less – neurospheres grown in absence of FGF-2 and EGF.

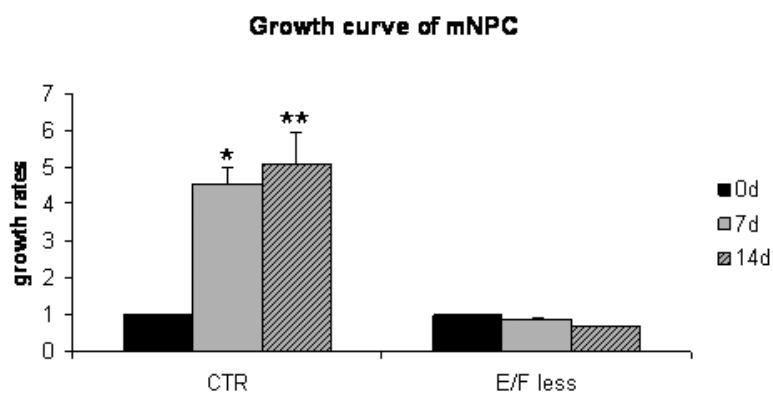
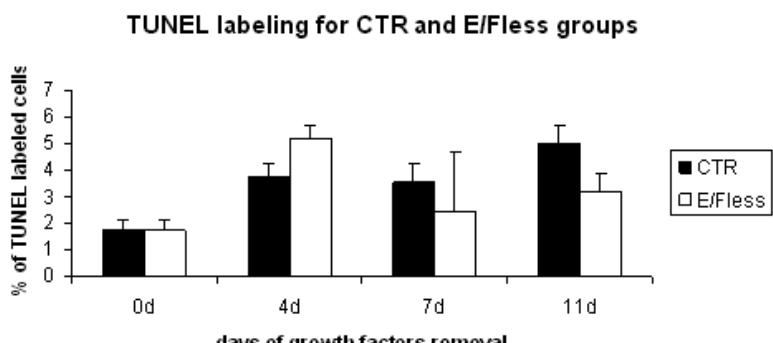


Fig. 2 – Analysis of mouse neurospheres growth during growth factor withdrawal. We found no significant increase in size during starvation of mNPC. In contrast, neurospheres from the CTR groups increased significantly in size during the experiment. After 7 days in culture, mNPC reach a *plateau* and they keep the same size until 21 days. Abbreviations: CTR – neurospheres submitted to regular conditions; E/F-less – neurospheres grown in absence of FGF-2 and EGF. Statistically significant differences in growth rates are represented by * ($n = 3$; $P < 0.005$).





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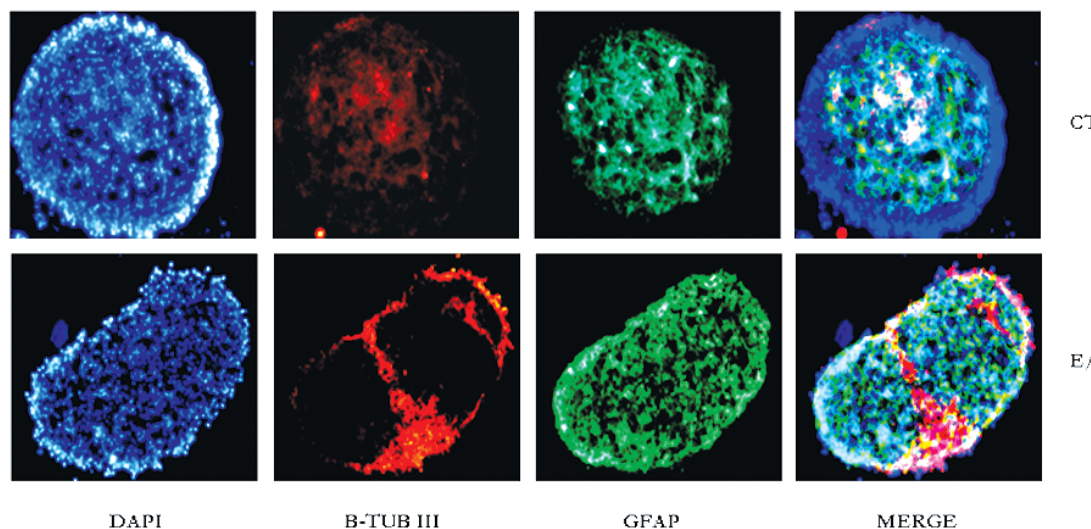


Fig. 4 – Immunolabeling of neurospheres slices for neurons (β -tubulin III – in red) and astrocytes (GFAP – in green) from the CTR and E/F-less groups of mNPC. GFAP positive cells were found across the whole neurosphere, whereas β -tubulin III positive cells were concentrated in the neurosphere core. A fusion of two neurospheres is shown for the (E/F-less) group. Despite the fusion, neurons are localized at the border of the neurospheres. Total nuclei were stained with DAPI (blue). 200X magnification.

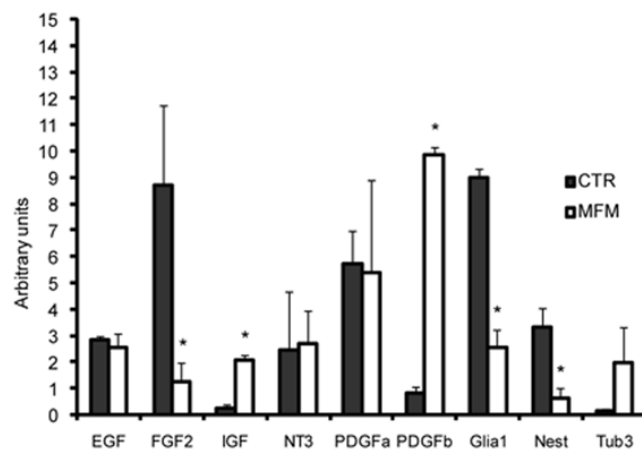


Fig. 5 – After 11 days of growth factor withdrawal, mNPC decrease the expression of FGF-2, GFAP and Nestin, and increase the expression of IGF-1 and PDGFb ($P < 0.05$).

cells from the core produce growth factors, maintaining these cells in an undifferentiated state. In the core of the growth factor deprived neurospheres, the smaller concentrations of such autocrine production of growth factors, as compared to exogenous administration in the

neurospheres (Figs. 1, 2). This represents a reasonable explanation for understanding the altered distribution of neuronal phenotypes (core *versus* shell) as a consequence of growth factor withdrawal.

Mouse neurospheres are known to produce



decreased the expression of *fgf-2*, *gfap* and *nestin*, and increased the expression of *igf-1* and *pdgfb* (Fig. 5). Taken together, these results suggest that, during EGF and FGF-2 removal, there is an alteration in the expression of growth factors and of neural specific proteins (*gfap*, β -*tubulin III* and *nestin*). When we removed growth factors from the culture media, cells stopped proliferating and started differentiating. Therefore, the decreased expression of *nestin*, a marker for stem cells or immature neural progenitors, was expected. Also, given that we encountered a tendency of increased transcripts of β -*tubulin III*, we suggest that growth factor removal preferentially lead to neuronal differentiation rather than glial differentiation, when compared to the standard culture media. In addition, as previously mentioned, *gfap* can be expressed by immature neural progenitors. In this way, if growth factor withdrawal induces differentiation of cells in suspension, the number of neural stem/immature cells should be expected to decrease. This, in part, could explain the difference in the level of differentiation and in the neuronal distribution in the neurospheres before and after growth factor removal.

Real-Time PCR experiments (Fig. 5) clearly demonstrate that endogenous production of growth factors shifts from FGF-2 to IGF-1/PDGFb upon growth factor withdrawal. Erickson and co-workers (2008) showed that the addition of low concentrations of IGF-1 was necessary for continued passaging and that NPC survived for long periods in culture without EGF or FGF-2 when IGF-1 was added to the media. It has been suggested that IGF-1 effects on NPC lifespan were mediated by means of its own tyrosine kinase receptor. The increased expression of PDGFb can be related to cell survival upon growth factor withdrawal (Ishii et al. 2008), since the PDGFb receptor signaling in neurospheres can prevent apoptosis. The hypothesis that the differential expression of *igf-1* and *pdgfb* in the E/F-less group is important to maintain cell survival is in agreement with the proportion of TUNEL positive cells (only 5%, for both groups). It is also known that PDGF influences the generation of neurons and glia during embryogenesis and in early postnatal life (Forsberg-Nilsson et al. 2003). Again,

media, may play a role in cell death and differentiation mechanisms in mNPC.

To conclude, the main findings of this work are that, under EGF and FGF-2 starvation, mNPC: i) stop growing; ii) show increased expression of *igf-1* and *pdgfb* that can promote cell survival; iii) have changes in the distribution of β -*tubulin III*+ cells according to the availability of growth factors; iv) induce differentiation of cells in suspension. These results may contribute to the understanding of EGF and FGF-2 mechanisms on NPC differentiation and are relevant for defining the ideal culture medium for neural progenitors expansion *in vitro*. In addition, these findings could be useful for therapies using NPC aiming at improved lifespan and integration of pre-differentiated cells after transplantation. Our findings suggest that depriving NPC of growth factors prior to grafting might enhance their chance to effectively integrate into the host.

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RESUMO

As terapias celulares para doenças neurológicas têm avançado e células precursoras neurais (NPC) surgem como candidatas ideais para o transplante de células neurais em muitas doenças. No entanto, trabalhos adicionais devem ser feitos para determinar o ambiente de cultivo ideal para a expansão *in vitro* das NPC, sem alterar sua plasticidade, e os mecanismos de sinalização celular do fator de crescimento epidérmico (EGF) e fator de crescimento de fibroblasto 2 (FGF-2) no crescimento, sobrevivência e diferenciação da neuroesfera. Nesse trabalho avaliamos NPC cultivadas na presença e na ausência de FGF-2 e EGF e mostramos que esses fatores de crescimento são responsáveis pela proliferação das NPC. Também foi demonstrado



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EGF e FGF-2. NPC de camundongo cultivadas em suspensão mostraram padrões de localização neuronal distintos (centro *versus* borda) tanto no grupo controle como no grupo sem EGF e FGF-2. Juntos, esses resultados mostram que a remoção de EGF e FGF-2 exerce importante ação na diferenciação de NPC e possivelmente contribui para melhor compreensão dos mecanismos envolvidos na diferenciação. Nossos achados sugerem que, privando as NPC de fatores de crescimento antes do transplante, talvez aumente as chances de que as células efetivamente se integrem ao hospedeiro.

Palavras-chave: células precursoras neurais, neuroesfera, fator de crescimento de fibroblasto 2, fator de crescimento epidérmico, diferenciação.

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