Chapter 71
PEDF Promotes Retinal Neurosphere Formation and Expansion In Vitro

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Abstract The retina is subject to degenerative conditions leading to blindness. Although retinal regeneration is possible in lower vertebrates, it does not occur in the adult mammalian retina. Retinal stem cell (RSC) research offers unique opportunities for developing clinical application for therapy. The ciliary body of adult mammals represents a source of quiescent RSC. These neural progenitors have a limited self-renewal potential in vitro but this can be improved by mitogens. Pigment Epithelium Derived Factor (PEDF), a member of the serpin gene family, is synthesized and secreted by retinal pigment epithelium (RPE) cells. We tested combinations of PEDF with fibroblast growth factor (FGF) during RSC growth to evaluate self-renewal and subsequent differentiation into retinal-like neuronal cell types. Medium supplemented with FGF + PEDF enhanced the RSC yield and more interestingly allowed expansion of the culture by increasing secondary retinal neurospheres after the 1st passage. This effect was accompanied by cell proliferation as revealed by BrdU incorporation. PEDF usage did not affect rod-like differentiation potential. This was demonstrated by immunofluorescence analysis of Rhodopsin and Pde6b that were found similarly expressed in cells derived from FGF or FGF + PEDF cultured RSC. Our studies suggest a possible application of PEDF in Retinal Stem Cell culture and transplantation.

71.1 Introduction

Many forms of blindness arise from photoreceptor degeneration and to date have no satisfactory solutions to rescue retinal tissues.

Mammalian eyes do not have a regenerative capability characteristic of lower vertebrates but recent evidences have demonstrated that ciliary body (CB), a

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structure analogous to the CMZ of lower vertebrates, contains retinal stem cells (RSC) (Moshiri et al. 2004; Nishiguchi et al. 2008; Tropepe et al. 2000; Ahmad et al. 2000; Coles et al. 2004; Inoue et al. 2005).

Multipotent retinal stem or progenitor cells can be isolated from the ciliary body of an adult mammalian retina using a neurosphere culture. Tropepe et al. have demonstrated that one out of 500 CB cells gives rise to a clonal aggregate called neurosphere consisting of ∼12,000 pigmented cells and that each neurosphere can generate six to eight daughter neurosphere colonies.

Although the presence and the function of retinal stem cells (RSCs) in vivo remains unknown RSCs proliferate in vitro; they differentiate into cell subtypes expressing markers of certain mature retinal neurons such as bipolar cells, photoreceptor cells or Mueller glia suggesting the possibility that these cells may represent a potential cell source in transplantation therapy for retinal diseases (Tropepe et al. 2000; Giordano et al. 2007). Differentiation is induced by plating the cells on an extracellular matrix substrate and exposing the cells to 1% Fetal Bovine Serum (FBS). In these conditions the percentage of cells that undertake a rod-like fate is about 30–40% as assessed by rhodopsin and the Pde6b co-expression. Growth of neurospheres with FGF without EGF before differentiation favors rod-like differentiation (Giordano et al. 2007).

Primary cultures of cells collected from the CB are in FBS free medium and the formation of neurospheres takes about 5–7 days. The number of neurospheres is dependent upon the growth factors to which cells are exposed, such as FGF or EGF. A limitation in the culture of RSC comes from low ability of these cells to be expanded in vitro. In fact, a single retinal neurosphere composed of about 12,000 cells upon passaging does not give rise to 12,000 new spheres but only an average of 3. Furthermore, no expansion of the culture has been obtained from the third passage (Giordano et al. 2007). Amelioration of the culture condition is therefore a fundamental aspect that needs to be addressed if we want to bring RSC to therapeutic applications.

Previous works have shown that pigment epithelium-derived factor (PEDF) secreted by the murine subventricular zone (SVZ) promotes self-renewal and activation of slowly dividing adult neural stem cells (NSC) in vitro (Ramirez-Castillejo et al. 2006). PEDF is a neurotrophic antiangiogenic factor initially purified from conditioned media of retinal pigment epithelial cells (Tombran-Tink et al. 1991; Becerra et al. 1995). PEDF is secreted from many different cells and can modulate cell cycle progression (Pignolo et al. 2003). Thus PEDF may have positive effects on RSC culture.

Here we show that the treatment of RSC with PEDF together with FGF increases the neurosphere yield and, ameliorates self-renewal in neurosphere passaging. We then tested whether PEDF could affect differentiation to rod-like fate. This improvement does not decrease the number of cells expressing rhodopsin and Pde6b. Moreover, cells differentiated from a FGF+PEDF culture did not express bipolar cell markers.

71.2 Materials and Methods

71.2.1 Retinal Stem Cell Isolation and Culture

All procedures on mice (including their euthanasia) were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with institutional guidelines for animal research. In this study we used C57BL/6 mice purchased from Charles River Italy (Calco, Italy) and housed them under standard conditions with a 12-hour light/dark cycle. We dissected eyes from 12-week-old C57BL6 mice in artificial cerebral spinal fluid (ACSF) containing 124 mM NaCl, 5 mM KCl, 100 nM CaCl2, 1.3 mM MgCl2, 26 mM NaHCO3 and 10 mM D-glucose. Eyes were cut in two hemispheres and the lens and the neural retina were carefully removed. The ciliary body was separated from the retinal pigment epithelium (RPE), treated with trypsin and hyaluronidase and the ciliary margin cells were scraped from the sclera according to the procedures described in the paper (Giordano et al. 2007). The isolated cells were grown in a serum free medium (0.6% glucose and N2 hormone mix in DMEM-F12) containing either 20 ng/ml basic FGF (FGF) supplemented with 2 μg/ml heparin (Sigma, Milan, Italy) or both FGF + 20 ng/ml PEDF (Chemicon). Cells were seeded at a concentration of 40,000 cells/ml and incubated for 6 days until floating spheres formed.

71.2.2 Single Sphere Passaging

A single sphere was placed in a microcentrifuge tube and incubated in enzyme solution (ACSF containing 1.33 mg/ml trypsin, 0.67 mg/ml hyaluronidase, 0.5 mg/ml collagenase type I-A, 0.5 mg/ml collagenase XI, 0.13 mg/ml kynurenic acid) for 1 h at 37°C. After centrifugation at 400*g* for 5 min the supernatant was replaced with 1 mg/ml of trypsin inhibitor in medium and the sphere was mechanically dissociated. Collected cells from each sphere were seeded into a 96-well plate in serum-free medium containing FGF or FGF+PEDF and incubated for 6 days.

71.2.3 Bromodeoxyuridine Labeling

Retinal floating spheres at 5 days of culture were treated with 10 mM bromodeoxyuridine (BrdU) for 3 h, washed with PBS, allowed to attach to a slide and fixed in 4% paraformaldehyde (PFA). Spheres were treated with 2 N HCl at 30°C for 30 min, placed in a 0.1 M borate buffer pH 8.5 for 15 min and then washed with PBS. Blocking was performed in a 3% bovine serum albumin (BSA), 1% glycine and 0.3% Triton-X 100 for 30 min at room temperature followed by incubation with 1:8000 anti-BrdU monoclonal antibody (Developmental Hybridoma,

Iowa City, IA) overnight at 4°C. Slides were washed with PBS, incubated with 1:1000 Alexa Fluor® 568 goat anti-mouse secondary antibody (Molecular Probes) for 1 h, washed and nuclei were labeled with 1 μg/ml Dapi (Roche) and treated with 2.5 mg/ml RNAse A at 37°C for 1 h. Slides were mounted with Moviol mounting solution (Sigma) and BrdU positive cells were counted in a stack of 20 images (5 μm) at a Leica laser confocal microscope system (Leica SP2, Wetzlar Gmbh Germany) of the CIGS University of Modena, Italy.

71.2.4 Retinal Stem Cell Differentiation

In differentiation experiments retinal floating spheres were plated on glass coverslips coated with extracellular matrix (ECM, Sigma). Cells were cultured in DMEM-F12 supplemented with either 20 ng/ml FGF and 2 μg/ml heparin or FGF + 20 ng/ml PEDF. The cells were allowed to proliferate and migrate out of the sphere over the course of 4 days. The medium was then replaced with 1% FBS (Gibco, San Giuliano Milanese, Italy) containing medium and cultured for 15 days.

71.2.5 Immunofluorescence

Cells grown on glass coverslips were fixed in 4% paraformaldehyde for 15 min at room temperature. Permeabilization and blocking was performed with 0.2% TritonX-100 and 3% bovine serum albumin (BSA) (Sigma, St Louis, MO) in PBS for 1 h followed by incubation with primary antibodies overnight at 4°C. Cells underwent five washes with PBS and then were incubated with fluorescent-conjugated secondary antibodies for 1 h at room temperature. Primary antibodies used were as follows: 1:1500 anti-Pkc-α rabbit polyclonal (Sigma), 1:100 anti-Pde6b rabbit polyclonal (ABCAM, Cambridge, UK), 1:100 anti-rhodopsin mouse monoclonal 1D4 (Sigma), 1:400 anti-G0α mouse monoclonal (Chemicon), 1:750 anti-syntaxin mouse monoclonal (Sigma), 1:1000 anti-calbindin D 28 K mouse monoclonal (Sigma). Secondary antibodies were as follows: 1:1000 Oregon Green® 488 goat anti-mouse (Molecular Probes, San Giuliano Milanese, and Italy) and 1:1000 Alexa Fluor® 568 goat anti-rabbit (Molecular Probes). Finally slides were mounted in moviol and analyzed using immunofluorescence microscopy Axiocam (Zeiss).

71.3 Results

71.3.1 PEDF Promotes Retinal Neurospheres Growth and Self-Renewal

Previous studies showed that retinal neurospheres had a limited ability to proliferate and to be expanded in vitro (Giordano et al. 2007; Gu et al. 2007). In all the

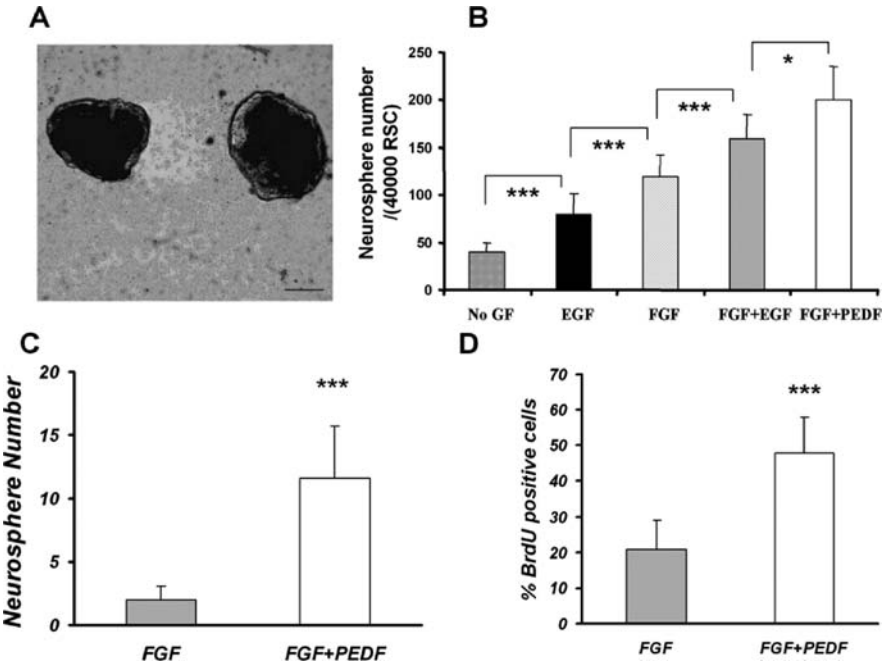

Fig. 71.1 Retinal neurospheres growth and proliferation. (**a**) RSCs grow clonally at low density to form neurospheres. Progenitor cells within the sphere proliferate for 7 days giving rise to a progeny of pigmented and non-pigmented cells. Scale bar, 50 μm. (**b**) Differences in neurosphere yield by culturing 40,000 cells with different growth factor combinations. (**c**) Secondary neurosphere number after single sphere passage grown with FGF or FGF + PEDF. The student t-Test was used. $^{***}p < 0.001$; ** $p< 0.01$; * $p< 0.05$. (**d**) Proliferation was evaluated in neurosphere by labeling with BrdU for 3 hrs at the 5th day of neurosphere formation. When cells were grown in FGF + PEDF medium a higher number of cells in proliferation (BrdU+) was counted

different conditions retinal neurospheres appeared with a similar morphology and pigmented (Fig. 71.1a). Cells were seeded at low density (40,000 cells/ml) in the growth medium and neurospheres were counted for each growth factor (GF) treatment. As shown in Fig. 71.1b, exposure to EGF doubled the floating sphere number compared to the cells plated in the absence of GF, while addictions of FGF increased this number of 3-fold. Moreover, the combination of FGF + EGF enhanced the neurosphere formation of 4 folds compared to the culture without GF. Because PEDF is a neurotrophic factor for some neural population, we set out to test whether the combination of PEDF + FGF enriches the neurosphere population and/or self renewal. The addiction of PEDF (20 ng/ml) to the FGF culture media resulted in a 60% increase of neurospheres when compared to FGF alone and 20% more neurospheres than FGF+EGF (n=3, $p < 0.01$).

Our previous studies have shown that EGF alone has a reduced ability to generate secondary neurospheres after passaging while cultures with FGF and EGF+FGF doubled the number of neurospheres at the first cell passage. In order to evaluate

whether the positive effect of PEDF was not limited to the primary culture in RSC but could be extended also to RSC self renewal we dissociated single primary generation neurospheres to single cells and re-plated them in the same culture media containing FGF+PEDF. The number of cells capable of generating a secondary neurosphere population increased in the presence of PEDF (Fig. 71.1c). We could measure a 5 time increase of passaged neurospheres when exposed to PEDF+FGF compared to FGF alone (n=4, $p < 0.001$). Altogether these data suggest that PEDF favors growth of retinal stem cells in primary cultures and enhances the chances of self-renewal in culture expansions.

71.3.2 Retinal Neurosphere Proliferation

A previous study carried on in our lab demonstrated that EGF in the culture medium of RSC favors proliferation during the first days of culture and then proliferation stops. On the other hand, FGF allows high proliferation to be maintained with time in culture. This observation may explain also the positive effect that FGF has on cell passaging. In order to evaluate if the increase in self-renewal properties we observed with PEDF was correlated with enhanced cell proliferation during the neurosphere growth, we labeled retinal spheres with BrdU at the sixth day of the retinal stem cell culture. BrdU positive ($BrdU^+$) and BrdU negative ($BrdU^-$) cells were counted and compared in FGF and FGF+PEDF culture conditions (Fig. 71.2). The presence of PEDF during neurosphere growth promoted cell proliferation more than

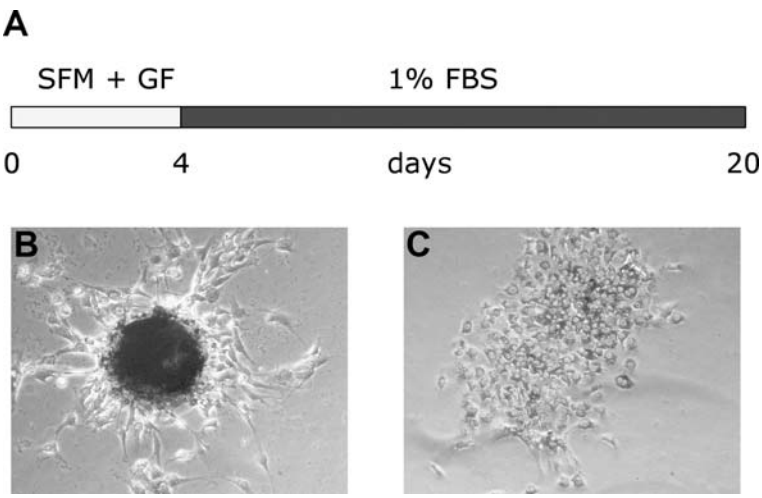

Fig. 71.2 RSC differentiation. (**a**) Schematic representation of the protocol for RSC differentiation: retinal neurospheres were plated onto an ECM substrate to let progenitor cells to exit for 4 days. The GF minimal medium was then replaced with 1% FBS and cells were differentiated up to 20 days. (**b**) Light microscope image of RSC at 4 days of differentiation where cells growing out of the dark sphere (centre) can be seen. (**c**) RSC at 7 days of differentiation

FGF alone. In particular, FGF gave an average of 20% of $BrdU^+$ cells within each retinal sphere while supplementation with PEDF increased this value up to 45% (Fig. 71.1d). This result indicates that the observed enhanced retinal neurosphere generation is accompanied by increased cell proliferation when RSCs are grown in presence of FGF+PEDF.

71.3.3 Differentiation of Retinal Cells Precursors from RSCs

The interest in the evaluation of RSC culture treatment correlates with the need of setting the favorable culture conditions to obtain rod photoreceptor differentiation. Several studies have indicated the possibility of using RSCs as a donor source.

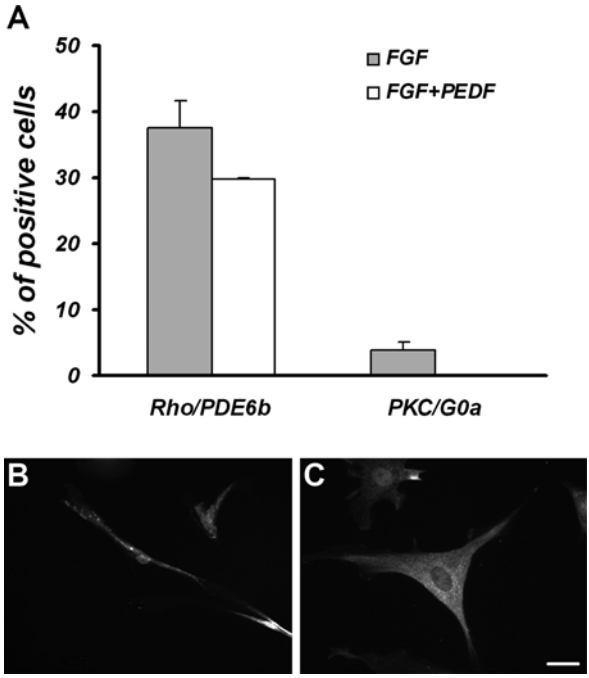

Fig. 71.3 Rod-like cell differentiation from PEDF treated retinal neurospheres. All the RSCs were differentiated in a 1% FBS culture medium. Differentiation was evaluated by immunofluorescence identifying rod-like cells and bipolar-like cells by co-expression of Rho/Pde6b and Pkcα/$G_0\alpha$ respectively. (**a**) *Grey bar* represents the amount of cells positive for markers derived from neurospheres grown in FGF medium; the *white bar* represents the amount of cells positive for markers derived from neurospheres grown in FGF + PEDF medium. Rod-like cells amount was similar when cells derived from spheres grown in either culture media while bipolar-like cells decreased when neurospheres were cultured in FGF + PEDF. The student t-Test was used for statistical analysis. *** $p< 0.001$; ** $p< 0.01$; * $p< 0.05$. (**b**) Co-immunolocalization of Rho and Pde6b markers in rod-like cells. (**c**) Co-immunolocalization of Pkc α and $G_0\alpha$, markers of bipolar cells. Scale bar in C represents 10 μm

We investigated the ability of differentiated RSCs to express typical markers of adult retinal cells. In our previous work we have evaluated the effect of different GF combinations on retinal cell differentiation. After 6 days of growth, retinal spheres were plated onto an extracellular matrix substrate for 4 days to allow progenitor cells to exit, attach and start differentiation (Fig. 71.2a–c). After 4 days the culture medium was replaced with media containing 1% FBS. Retinal progenitor cells (RPCs) were cultured up to 20 days and then underwent expression analysis by immunofluorescence labeling. Rod photoreceptor-like cells were elongated and defined by the co-expression of specific rod markers: Rhodopsin and Pde6b (an example of Rhodopsin distribution is shown in Fig. 71.3b). Rod-like cells were also characterized by smaller cell size accompanied by smaller nuclei compared to the Rho/Pde6b negative cells. Bipolar-like cells were identified by co-expression of $G_0\alpha$ and Pkcα (an example of Pkcα distribution is shown in Fig. 71.3c). Our culture condition was also favorable for expression of horizontal and amacrine markers like calbindin and syntaxin respectively (data not shown). Generation of neurospheres in the presence of FGF+PEDF did not significantly alter the number of rod-like cells that reached 30–40% of the total cultured cells (Fig. 71.3a). In turn, we were not able to detect bipolar-like cells in the FGF+PEDF culture while 5% of cells treated with FGF only were positive to $G_0\alpha$/Pkcα.

Altogether, results on retinal cell differentiation indicate that PEDF does not significantly affect rod-like differentiation.

71.4 Discussion

Few cells in the mammalian adult ciliary body have the ability to proliferate when subjected to the appropriate stimuli (Tropepe et al. 2000; Ahmad et al. 2000; Zhao et al. 2002). This capability, which resides in less than 1% of the ciliary body cells, can be ameliorated with exposure to growth factors in the culture media. In our previous work we have demonstrated that addiction of FGF + EGF gave the highest neurosphere yield and that this property is maintained also over daughter neurosphere generation after sphere passage (Giordano et al. 2007). Despite the enhanced neurosphere yield, the presence of EGF decreased the rod-like cell number during RSC differentiation and increased the bipolar-like marker expression. This latter result prompted us to investigate alternative GF combinations in order to maximize the RSC self-renewal without affecting the photoreceptor-like cell amount during the following differentiation. Here we report that PEDF in combination with FGF ameliorates the neurosphere formation, likewise acting on the self-renewal capability of the RSCs. This property already described by PEDF on NSC derived from the adult SVZ (Ramirez-Castillejo et al. 2006), was also confirmed during retinal neurosphere passaging. Self-renewal involves both proliferation and maintenance of undifferentiated state, although this process is still poorly understood at molecular levels. We propose here that PEDF might be a modulator during cell division likely promoting generation of two identical stem cells or one stem cell and one committed progenitor instead of two committed progenitor cells. Unlike NSC,

(Ramirez-Castillejo et al. 2006) we find that PEDF has a mitogen effect on RSC as indicated from BrdU incorporation during neurosphere growth. This might be due to cell-intrinsic mechanisms (Arsenijevic 2003) or due to the lack of some external cues such as growth promoting NSC culture conditions.

Upon differentiation, PEDF does not interfere with rod-like cell marker expression giving a similar amount of ∼35% to those cells differentiated in the FGF only medium. However, the true identity of photoreceptor-like cells with further molecular and functional analysis remains to be investigated.

Although PEDF has no influence on the rod-like cells number, it causes a lack in bipolar-like cell amount. This effect suggests a possible role of PEDF on Chx10 gene regulation since it has been reported in the involvement of this transcription factor in bipolar cell differentiation (Livne-Bar et al. 2006; Kokkinopoulos et al. 2008). The fact that loss of bipolar-like cells is not accompanied by an increase in other interneuron or rod-like cells may lead to enrichment in progenitor cells still capable of being addressed toward a diverse retinal cell fate, however, this needs to be documented by further studies.

Taken together, these findings indicate that PEDF may contribute to ameliorate RSC expansion, offering a source of alternative therapy in regenerative medicine.

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