Fibroblast growth factor and epidermal growth factor differently affect differentiation of murine retinal stem cells in vitro

Francesca Giordano, Anna De Marzo, Francesco Vetrini, Valeria Marigo

1 Telethon Institute of Genetics and Medicine, Naples; 2 Department of Biomedical Sciences, University of Modena and Reggio Emilia, Modena, Italy

Purpose: The developmental processes that mediate differentiation from retinal stem cells (RSC) to different retinal neuronal types remain unclear. During retinal development, progenitor cells modify expression of growth factor (GF) receptors and their differentiation potentials. Similarly, RSC in culture may exhibit alternative molecular characteristics in response to different GF stimuli.

Methods: RSC were purified from the adult ciliary margin and exposed to fibroblast growth factor (FGF), epidermal growth factor (EGF), or FGF+EGF. Proliferation was analyzed by bromodeoxyuridine (BrdU) labeling. Differentiation was evaluated by immunofluorescence with antibodies recognizing specific markers of different retinal cell types.

Results: In the absence of GF stimuli, RSC in culture expressed FGFR1, similar to early progenitors in vivo. Treatment with GFs up-regulated the expression of both fibroblast growth factor receptor 1 (FGFR1) and epidermal growth factor receptor (EGFR). Exposure to either FGF, EGF, or FGF+EGF strongly affected retinal stem cell-renewal and differentiation. Specifically, expression of progenitor/stem cell markers and stem cell-renewal was higher in the presence of FGF than in that of EGF. FGF favored differentiation of RSC into photoreceptor-like cells. Finally, we showed that the treatment of the primary culture with FGF+EGF imprinted the cells and limited plasticity in subsequent differentiation.

Conclusions: We provide evidence that conditions of the primary culture have a strong influence on cell-renewal and differentiation potentials of RSC.

The retina is a highly organized laminar structure in which cells are born in a sequential, temporally defined order. It has been suggested that characteristics of retinal progenitors change during differentiation. Early progenitors are able to differentiate into all retinal neuronal and glial cell types while later progenitors are unable to give rise to early born retinal neurons such as ganglion cells and cones, but generate late born cells [1]. This competence appears to be due both to intrinsic characteristics and external stimuli. Among the intrinsic features are relative expressions of fibroblast growth factor receptor 1 (FGFR1) and EGFR. Retinal progenitor cells in early embryonic retina normally express higher levels of FGFR1 and lower levels of EGFR than progenitor cells in later retina. The differential expression of FGFR1 in retinal progenitor cells at distinct developmental stages has been proposed to be associated to signaling thresholds affecting differentiation [2].

Retinal progenitors can be purified from the embryonic retina and induced to differentiate into photoreceptors and other retinal neuronal cell types [3]. Stem cells have also been identified in the marginal region of the adult eye, and retinal stem cells (RSC) can be derived and cultured in vitro from the adult rodent and human ciliary body [4-6] and iris [7]. RSC are purified by enzymatic and mechanical dissociation and grown as pigmented neurospheres in suspension in minimal medium without serum but supplemented with growth factors, such as fibroblast growth factor (FGF) and epidermal growth factor (EGF). These cells are able to self-renew because they clonally generate new retinal spheres. When retinal spheres are allowed to grow on a laminin substrate in the presence of fetal bovine serum (FBS) they undergo differentiation into different retinal neuronal and glial cell types [4-6]. It is still unclear how much primary culture conditions have an effect on RSC self-renewal and differentiation potential. Das et al. suggested that RSC have an early progenitor receptor code with higher levels of FGFR1 than EGFR [8]. It is therefore reasonable that preparation and culture of retinal spheres in the presence of either FGF or EGF may affect their differentiation competence.

We assessed proliferation and differentiation potentials of RSC treated with either FGF, EGF, or FGF+EGF. Here we show that the different growth factors strongly affect retinal stem cell proliferation kinetics, self-renewal, and differentiation potential. Interestingly, GF treatment of a primary retinal sphere also influences reprogramming during generation of secondary retinal spheres and limits their differentiation potential.
METHODS

Retinal stem cell 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. Mice in this study were C57Bl6, purchased from Charles River Italy (Calco, Italy) and housed 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) that containing 124 mM NaCl, 5 mM KCl, 100 nM CaCl₂, 1.3 mM MgCl₂, 26 mM NaHCO₃, and 10 mM D-glucose. Eyes were halved, and the lens and the neural retina were carefully removed. The ciliary margin was separated from the retinal pigment epithelium (RPE), and the cornea and the ciliary margin cells were scraped from the sclera after incubation in dispase (BD Biosciences, Milan, Italy) for 10 min at 37 °C and in enzyme mix (1.33 mg/ml trypsin, 0.67 mg/ml hyaluronidase, and 0.13 mg/ml kynurenic acid in ACSF) for 10 min at 37 °C. Cells were mechanically separated and centrifuged at 1500 rpm for 5 min. The enzyme solution was removed and replaced with serum free media containing 1 mg/ml trypsin inhibitor (Invitrogen, San Giuliano Milanese, Italy). Cells were further dissociated until single cell suspension and then centrifuged again. The supernatant was replaced with serum free medium (0.6% glucose and N2 hormone mix in DMEM-F12) containing either 20 ng/ml basic FGF supplemented with 2 µg/ml heparin (Sigma, Milan, Italy), or 20 ng/ml EGF, or both FGF and EGF. The cells were seeded at a concentration of 40,000 cells/ml and incubated for 3-7 days until floating spheres formed.

In differentiation experiments, retinal floating spheres were plated on eight well glass slide that was coated with extracellular matrix (ECM, Sigma) in DMEM-F12 supplemented with either 20 ng/ml FGF or 20 ng/ml EGF or FGF+EGF. The cells were allowed to proliferate and migrate over the course of four days. The medium was then replaced with 1% FBS (Gibco, San Giuliano Milanese, Italy) containing medium.

In vitro passaging: Single retinal floating spheres treated with either FGF, EGF, or EGF+FGF were collected and incubated in enzyme solution (in ACSF containing 1.33 mg/ml trypsin, 0.67 mg/ml hyaluronidase, and 0.13 mg/ml kynurenic acid in ACSF) for 1 h at 37 °C. Cells were mechanically separated and centrifuged at 1500 rpm for 5 min. The enzyme solution was removed and replaced with serum free media containing 1 mg/ml trypsin inhibitor (Invitrogen, San Giuliano Milanese, Italy). Cells were further dissociated until single cell suspension and then centrifuged again. The supernatant was replaced with serum free medium (0.6% glucose and N2 hormone mix in DMEM-F12) containing either 20 ng/ml basic FGF supplemented with 2 µg/ml heparin (Sigma, Milan, Italy), or 20 ng/ml EGF, or both FGF and EGF. The cells were seeded at a concentration of 40,000 cells/ml and incubated for 3-7 days until floating spheres formed.

In reprogramming experiments, EGF+FGF treated primary spheres after the first passage were incubated with FGF, EGF, or EGF+FGF.

Immunofluorescence: Cells were fixed in 4% paraformaldehyde for 15 min at room temperature. They were then permeabilized with 0.2% TritonX-100 in 10% goat serum and incubated with primary antibodies in (PBS) overnight at 4 °C. Cells underwent five washes with PBS, then were incubated with fluorescent-conjugated secondary antibodies for 1 h at room temperature. Primary antibodies used were as follows: 1:400 anti-nestin mouse monoclonal (Chemicon, Chondlers Ford, UK), 1:1500 anti-Pck-c-rabbit polyclonal (Sigma), 1:100 anti-Pde6b rabbit polyclonal (ABCAM, Cambridge, UK), 1:10,000 anti-rhodopsin mouse monoclonal RETP1 (Sigma), 1:400 anti-GFAP rabbit polyclonal (Sigma), 1: 400 anti-Gs6 mouse monoclonal (Chemicon), 1:400 anti-G0gt mouse monoclonal (Chemicon). Secondary antibodies were as follows: 1:1000 Oregon Green® 488 goat anti-mouse (Molecular Probes, San Giuliano Milanese, Italy) and 1:1000 Alexa Fluor® 568 goat anti-rabbit (Molecular Probes). Fluorescent cells in single spheres were counted on 20 image stacks (5 µm) generated by a Leica laser confocal microscope system.

Bromodeoxyuridine labeling: Retina floating spheres at three, five, and seven days of culture and adherent progenitors at four days of culture were treated with 10 mM bromodeoxyuridine (BrdU) for 3 h and then washed with PBS and fixed in 4% paraformaldehyde (PFA). Cells were treated with 2N 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 10% goat serum, 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, then incubated with 1:1000 Oregon Green® 488 goat anti-mouse secondary antibody (Molecular Probes) for 1 h, washed and nuclei were labeled with 50 µg/ml propidium iodide and 2.5 mg/ml RNAse A at 37 °C for 1 h. Slides were mounted with Vectashield (Vector Laboratories, Segrate, Italy), and BrdU positive cells were counted in a stack of 20 images (5 µm) at a Leica laser confocal microscope system.

Real-time polymerase chain reaction: Total RNA was extracted from floating neurospheres and adherent cells using RNeasy MiniKit (Qiagen, Milan, Italy) according to the manufacturer’s instructions. The same amount of cDNA for each treatment was synthesized using Superscript II (Invitrogen) and random primers. Real-time PCR was carried out with the GeneAmp 7000 Sequence Detection System (Applied Biosystems). The PCR reaction was performed using cDNA, 12.5 ml SYBR green master mix (Applied Biosystems) and 400 nM primer for each gene (see Table 1). Water was added to a final reaction volume of 25 ml. The PCR conditions were as follows: preheating at 50 °C for 2 min and 95 °C

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’→3’)</th>
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<tr>
<td>CYCD1</td>
<td>F: CTGCTGCAAATGGAATGTGCTT</td>
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<td></td>
<td>R: GCCAGGTTCCTGACTTGAGC</td>
</tr>
<tr>
<td>CYCD1</td>
<td>F: CTGCCTAAGGTGCAATCCCA</td>
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<tr>
<td></td>
<td>R: AACTTCAGGCTCTGGCAGC</td>
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<tr>
<td>FGFR1</td>
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<td>R: GCCGGGCTATCCGACTCATC</td>
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Table 1. Real time polymerase chain reaction primers
Figure 1. Proliferation of retinal spheres. 

A-C: Confocal images of bromodeoxyuridine (BrdU) staining (green) of retinal spheres cultured for seven days in the presence of either fibroblast growth factor (FGF; A) or epidermal growth factor (EGF; B), or FGF+EGF (C). Nuclei were stained with propidium iodine (red). There was a proliferation of cells at the seventh day of culture when retinal spheres were grown with FGF. Scale bar represents 60 µm. 

D: Percentage of BrdU+ cells counted in spheres at the third day, fifth day, or seventh day of culture (D3, D5, and D7). Values are shown as percentage of BrdU+ stained nuclei versus the total number of nuclei and are the average of BrdU+ cells counted in five spheres of three different experiments. The Student’s t-test was performed. Asterisk (*) indicates p<0.05, and double asterisk (**) represents p<0.01. Significance was calculated by comparing spheres grown with FGF to spheres grown with either EGF, or FGF+EGF. 

E: Quantitative analysis of Cyclin D1 mRNA expression in spheres cultured for seven days. Spheres grown in the presence of (FGF, EGF or FGF+EGF) expressed a significant higher level of Cyclin D1 than grown in the absence of growth factor (-GF), but there was no significant measured difference among cultures with different GFs.
for 10 min; 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Quantification results were expressed in terms of the cycle threshold (Ct). All real-time quantitative PCR reactions were run in triplicate and the Ct values were averaged from three independent samples. The S26 gene was used as an endogenous control (reference marker). Differences between the mean Ct values of each gene and those of the reference gene were calculated as $\Delta$Ct=$C_{\text{gene}}-C_{\text{S26}}$ and represented as $2^{-\Delta Ct}$ values.

RESULTS

Progenitor markers and self-renewal of retinal stem cells in culture: Previous studies have shown different responsiveness of RSC to EGF and FGF [8]. In order to better characterize these observations at the molecular level, we tested different in vitro culture conditions of retinal spheres derived from the murine adult ciliary margin. We generated primary spheres with minimal medium in the presence of either FGF, EGF, or FGF+EGF. We compared growth competence of cells exposed

![Figure 2. Expression of progenitor markers, FGFR1 and EGFR, in retinal spheres. A-C: Spheres grown with either fibroblast growth factor (FGF; A) or epidermal growth factor (EGF; B) or FGF+EGF (C) were stained with antibodies anti-nestin (green) or anti-Pkcα (red). Some cells co-expressed nestin and Pkcα (yellow). Scale bar represents 60 µm. D-F: In these panels, the percentage of nestin (D), Pkc-α (E), and GFAP (F) positive cells are given after treatment with either FGF (red bar), EGF (yellow bar) or FGF+EGF (green bar). Values are shown as percentage of stained cells versus the total number of nuclei and are the average of positive cells counted in five spheres of three different experiments. G: Percentage of cells in proliferation bromodeoxyuridine (BrdU+) that were labeled with anti-Pkc-α (Pkcα+) in spheres grown with either FGF (red bar), EGF (yellow bar), or FGF+EGF (blue bar). More than 50% of cells in proliferation expressed Pkc-α. H-I: Quantitative analysis of FGFR1 mRNA (H) and EGFR mRNA (I) expression in spheres cultured for seven days in the absence of any growth factor (GF; blue bar) or in the presence of either FGF (red bar), EGF (yellow bar) or FGF+EGF (blue bar). Expression of both receptors was increased by treatment with GFs. FGFR1 receptor was similarly expressed when spheres were grown with FGF alone and FGF+EGF. Differently EGFR expression showed an additive effect when spheres were cultured with FGF+EGF. The Student t-test was employed, and significance was calculated by comparing spheres grown with FGF to spheres grown with either EGF or FGF+EGF. Asterisk (*) represents p<0.05, double asterisk (**) indicates p<0.01, and triple asterisk (***) indicates p<0.001.

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to different growth factors and found that treatment with both
growth factors gave rise to spheres larger in diameter than
spheres treated with either FGF or EGF (Figure 1A-C). When
we evaluated proliferation by using BrdU labeling, we found
that proliferation was strongly stimulated in the first days of
culture by the presence of EGF with 15% of BrdU positive
cells at the fifth day of culture. This probably accounts for
the larger diameter of the spheres at D7 (Figure 1D). However,
growth kinetics were delayed when cells were treated with
FGF alone, showing significantly lower numbers of prolifer-
ating cells at the fifth day of culture (9%) but 20% of BrdU
positive cells after seven days of culture (Figure 1A,D). It is
possible that spheres would increase in size with longer time
in culture with FGF. Interestingly, cells exposed to either EGF
or FGF+EGF have a sharp drop in proliferation after five days
in culture. Proliferation was also evaluated by Cyclin D1 ex-
pression at day seven of culture. Spheres treated with GF ex-
pressed higher levels of Cyclin D1 compared to spheres not
exposed to GF (Figure 1E).

We then analyzed expression of markers of neuronal stem
cells and progenitors. Seven days of culture in the presence
of FGF alone or together with EGF allowed higher expression
of nestin and GFAP than with EGF alone (Figure 2D,F). Most
cells positive to nestin and GFAP also expressed Pkc-α (Fig-
ure 2A-C; data not shown), a marker previously reported, and
confirmed by our immunofluorescence analysis, to label only
bipolar cells in the adult retina [9]. Here we found that Pkc-α
was also expressed in retinal spheres and showed partial co-
localization with nestin (Figure 2A-C). Further, we confirmed
that Pkc-α labels proliferating progenitors by double staining
with BrdU and anti-Pkc-α (Figure 2G). Otherwise, exposure
to only EGF limited the number of cells expressing stem cell/
progenitor markers.

Given the differential expression of EGFR and FGFR1
by retinal progenitors at different developmental stages [10],
we considered whether different treatments affected expres-
sion of the two receptors. As previously reported, we found
that in the absence of GF, RSC express mostly FGFR1 (Fig-
ure 2H-I blue bars) [8]. After seven days in culture FGFR1
mRNA appeared to be induced by treatment with GFs and
relatively more by the presence of FGF in the culture medium
(Figure 2H). A similar result was observed when EGFR ex-
pression was analyzed (Figure 2I).

Self-renewal was assessed by dissociation of the primary
retinal spheres and re-plating in GF medium. Retinal spheres
treated with EGF alone showed a poor ability to give rise to
secondary spheres. Cultures with FGF alone or FGF+EGF
doubled the number of retinal spheres at the first passage, but
this characteristic was lost in the following passages (Figure
3A). In order to determine if the decrease in self-renewal abil-
ity was associated with a reduction in cell proliferation of sec-
ondary spheres, we labeled secondary retinal spheres with
BrdU. The percentage of proliferating cells was only slightly
reduced compared to primary retinal spheres (Figure 3B) and
confirmed that the presence of EGF in the culture medium
negatively affected cell proliferation.

Altogether, these data showed that EGF does not favor
expression of stem cell markers and limits RSC renewal and
proliferation.

**Differentiation potentials of retinal stem cells in culture:**
The interest in the evaluation of RSC culture treatments was
derived from the search of favorable conditions to obtain rod
photoreceptor differentiation. We therefore allowed primary
retinal spheres to attach to an ECM substrate and differentiate
without using specific treatments other than 1% FBS. Cells
were treated with the different GFs during the first four days
in culture to facilitate their growth out of the spheres. At this
time point both FGF and EGF similarly induced proliferation
as evaluated by BrdU incorporation or cyclin D1 expression
(data not shown).
Differentiation of cells derived from RSC treated with the different GFs was evaluated by immunofluorescence analysis at different times during differentiation. We decided to analyze in detail differentiation of rods and compare this to differentiation of bipolars, because these two retinal cell types differentiate in vivo during the same time window. Under proper condition, progenitors have also been shown to be able to change their fate from rods to bipolars [9]. Our culture conditions were appropriate for differentiation into cells expressing several retinal neuronal markers such as horizontal-like cells (positive to Calbindin, Figure 4I), amacrine-like cells (positive to Syntaxin, Figure 4J), and Müller glia-like (positive to GFAP and GS6, Figure 4K). Rod photoreceptor-like cells were identified by co-expression of two cell-specific markers: rhodopsin (Rho) and Pde6b (Figure 4A-D). An elongated shape of the cells with accumulation of Rho at the peripheral region was observed in prolonged cultures (Figure 4B,D, arrow). Bipolar-like cells were defined as positive to Pkc-α and to G0α, a marker of ON bipolars [11], but nestin negative (Figure 4E-H). As previously shown, in our hands Pkc-α is co-expressed with stem cell/progenitor markers in retinal spheres. G0α was never observed after four days in culture but turned on in prolonged cultures (Figure 4H, arrow). Finally, co-expression of nestin and Pkc-α characterized retinal progenitors (Figure 4E-F, arrows). Progenitors decreased over differentiation time with all treatments, and EGF appeared to favor this phenomenon as assessed by nestin and Pkc-α expression (Figure 5A). Generation of retinal spheres in the presence of FGF increased the number of cells differentiating into rod-like cells and, interestingly, four days in differentiating condition allowed 25% of cells to express rhodopsin and Pde6b (Figure 5B). The percentage of rod-like cells slightly increased over time, reaching 30% after 18 days of culture (Figure 5B). Concomitantly, cells acquired an elongated shape (Figure 4C-D). Bipolar-like cell fate was favored

Figure 4. Differentiation into retinal-like neurons. Retinal stem cell were allowed to differentiate for four days (D4) or 18 days (D18) on an extracellular matrix (ECM) substrate. A-D: Rod-like cells were analyzed by co-localization of two different markers: Rhodopsin (Rho, green) and Pde6b (red). The arrow in D indicates a cell that co-expressed the two markers and in which Rho accumulated at the periphery of the cell. Scale bar in A and B represents 20 µm. Scale bar in D represents 10 µm as for all images from C-K. E-F: Progenitors and bipolar-like interneurons were distinguished by immunolabeling with antibodies anti-nestin (red) and anti-Pkcα (green). Progenitors co-expressed the markers nestin and Pkc-α (arrows). G-H: Bipolar-like cells were analyzed by co-localization of two different markers: Pkcα (red) and G0α (green). Arrow indicates a cell co-expressing the two markers after 18 days in differentiation. G0α was not expressed in cells after four days of differentiation. I: Immunolocalization of Calbindin, a marker for horizontal cells. J: Immunolocalization of Syntaxin, a marker for amacrine cells. K: Müller glia cells were analyzed by co-localization of two different markers: Gfap (red) and GS6 (green). Arrow indicates a cell that co-expressed the two markers. L: quantitative analysis of FGFR1 and EGFR mRNA expression in retinal progenitors cultured for 18 days in the presence of fibroblast growth factor (FGF; red bar), epidermal growth factor (EGF; yellow bar), FGF+EGF (green bar) indicates that expression of both receptors is relatively lower in cultures with EGF. The Student t-test was used, and significance was calculated by comparing spheres grown with FGF to spheres grown with either EGF or FGF+EGF. An asterisk (*) indicates p<0.05, and a double asterisk (**) denotes p<0.01.
by preparation of retinal spheres with EGF. In this condition we obtained 24% of cells expressing Pkc-α and turning off nestin within four days of differentiation (Figure 5D). We also observed that over time in culture, the percentage of cells expressing Pkc-α decreased. To better characterize cells after 18 days of differentiation, we analyzed expression of FGFR1 and EGFR. FGFR1 mRNA was only slightly lower in cultures derived from spheres treated with EGF than with FGF alone, while EGFR mRNA was significantly higher in cultures exposed to FGF alone (Figure 4L).

We finally tested whether RSC were able to reprogram themselves during in vitro passaging. Toward this aim we set primary cultures of retinal spheres in the presence of FGF+EGF. Cells were left in culture for six days to allow spheres to form, then were dissociated and plated in stem cell conditions with either FGF, EGF, or FGF+EGF. Newly formed retinal spheres (secondary retinal spheres) were seeded on an ECM substrate and permitted to differentiate as done for primary cultures. The initial treatment with both GFs had a strong effect on differentiation potentials of the cells (Figure 5C,E). Specific differentiation effects of FGF and EGF stimuli were attenuated in the secondary cultures. Treatment of the secondary culture with FGF gave rise to less photoreceptor-like cells (21.7±2.5 versus 31.0±2.3) and more bipolar-like cells (8.3±1.5 versus 4±0.85) than cells that were exposed to FGF from the primary culture. On the contrary, exposure of the primary culture to both GFs did not affect EGF induction of rod photoreceptor-like cells (7.3±1.5 versus 11.5±1.6; Figure 5C,E). Of note is the observation that primary and secondary cultures exposed to FGF+EGF showed similar differentiation potentials with any treatment. The average number of cells expressing rod cell markers is similar in all conditions and not significantly

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Figure 5. Differentiation time course of rod-like and bipolar-like cells. A, B, D: Primary retinal spheres were treated with either fibroblast growth factor (FGF), epidermal growth factor (EGF), or FGF+EGF and then differentiated on an extracellular matrix (ECM) substrate. Differentiation was evaluated by immunofluorescence identifying markers for either progenitors (A, positive to nestin and Pkc-α) or rod-like cells (B, positive to rhodopsin and Pde6b) or bipolar-like cells (D, marked by expression of Pkc-α and G0α). Cells were analyzed after four (white bars), 13 (black bars), and 18 (red bars) days of culture. Progenitors decreased in all culture conditions while induction of rod-like cells was favored by FGF and bipolar-like cells by EGF. C, E: Primary retinal spheres were grown with FGF+EGF. At the first passage, cells were exposed to either FGF, EGF, or FGF+EGF to generate secondary retinal spheres. Secondary retinal spheres were then allowed to differentiate for 18 days. Blue bars represent percentage of rod-like cells (C) and bipolar-like cells (E) derived from secondary spheres. Red bars represent percentage of rod-like cells after 18 days of differentiation of primary spheres as shown in B and percentage of bipolar-like cells after 18 days of differentiation as shown in D. The Student t-test was used, and significance was calculated by comparing Rho/Pde6b+ or Pkc/G0α+ cells differentiated from primary cultures to those derived from secondary cultures. Asterisk (*) indicates p<0.05, and double asterisk (**) marks p<0.01.
different from primary cultures exposed to FGF+EGF (about 20% in Figure 5C). Similarly, we observed that the primary treatment with FGF+EGF gave rise to a similar percentage of cells choosing the bipolar-like fate when treated with the three different conditions (about 8% in Figure 5E).

**DISCUSSION**

The aim of this work was to better characterize RSC derived from the adult ciliary margin and define their differentiation potential. We primarily wanted to analyze how FGF and EGF affect proliferation, self-renewal, and differentiation of RSC. We started by characterizing molecular features and self-renewal of RSC in culture. We report that treatments with distinctive GFs differently affect cell proliferation of the retinal spheres. Proliferation in the first days of culture is favored by the presence of EGF in the culture medium but then drops off after seven days. This may affect self-renewal as suggested by our data showing that cells treated with EGF alone have minimal ability to form new retinal spheres after passing. The drop in recovery of retinal spheres after the second passage is not easily explained. The percentage of proliferating cells in secondary retinal spheres remains similar to primary spheres, but we probably reduce the percentage of stem cells compared to progenitors that are not able to self-renew in vitro. Our data are in agreement with previous reports that showed a similar low recovery after the second passage [12].

Our analysis points to an influencing effect of the primary culture conditions on the differentiation potential of retinal progenitors. Treatment with FGF favors rod-like differentiation. Time course experiments showed that within eight days the number of cells expressing Rhodopsin and Pde6b reached 30% and was maintained during the subsequent 10 days in culture. In addition, a longer time in culture allowed the cells to acquire an elongated shape and to accumulate rhodopsin at the tip of the cell. This suggests that FGF positively acted on rod-like differentiation while EGF had a negative effect by limiting the number of cells expressing rod markers. It is not likely that EGF reduced cell viability because the number of cells turning on rod cell markers continuously increased over time in culture (see Figure 5B). The inducing activity of FGF is also supported by the experiment in the presence of FGF+EGF in which the percentage of cells that differentiated into rod-like cells after four to eight days of differentiation is higher than in EGF treated cells. Previous reports suggested a positive role of FGF in rod differentiation and survival, but not the negative effect of EGF [13].

The choice to become bipolar-like cells is favored by EGF that, however, may not be sufficient for the maintenance of this cell type. Cells expressing bipolar cell markers decreased over time in culture. Because of the late expression of the G0α marker, it is possible that not all cells positive to Pkc-α but negative to nestin will eventually complete their bipolar-like differentiation. Our data also suggested that expression of Pkc-α in in vitro cultured retinal cells is not strictly related to expression of this marker in bipolar cells but also labels progenitor cells. The number of nestin-Pkc-α double labeled cells is higher in FGF-treated cells than in EGF-treated cells, while the positive effect of EGF on bipolar-like differentiation is shown by the high percentage of G0α-Pkc-α double labeled cells. FGF appeared to oppose bipolar-like differentiation as can be seen by the low percentage of cells expressing G0α-Pkc-α in cultures treated with both FGF and EGF or FGF alone.

The differential role of EGF and FGF in differentiation may be due to their effect on progenitors and not directly on stem cells. We should keep in mind that only a small number of cells in each retinal sphere are real stem cells (after dissociation, not all cells form new retinal spheres as shown by our self-renewal test), and progenitors may have chosen alternative commitments in response to different GFs. Cells within a few days on ECM substrate start to turn off progenitor markers like nestin and undertake their fate. We cannot exclude that these cells are still malleable and able to change their choice when properly instructed.

Our study provides evidence that exposure of freshly dissociated cells from the ciliary margin to different GFs affected their differentiation potentials in subsequent passages. Cells that were treated in the primary culture with FGF+EGF gave rise to a defined percentage of rods and bipolar cells even if only one of the GFs was present in the secondary culture. This was an unexpected result. Even if GFs can induce progenitor pre-commitment, cells after dissociation and growth, as secondary spheres with either FGF or EGF, should behave like freshly harvested stem cells. Our observations suggest that stem cells in culture are affected by different GFs. Further studies are needed to define the molecular events underlying this phenomenon.

Our study highlights the importance of GFs as favoring conditions in vitro differentiate retinal stem cells. This is the first step toward the definition of optimal cell culture conditions in vitro generate rod photoreceptors that may be employed in transplants of degenerating retinas. As recently suggested [14], a proper differentiation of RSCs into photoreceptor precursors will be fundamental to obtain graft integration and proper differentiation of transplanted cells. Future studies will focus on the identification of more specific inducing factors to be used in differentiating cultures and to allow a higher percentage of cells to undertake the rod pathway.

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