

## Different levels of epidermal growth factor signaling modifies the differentiation of specific cell types in mouse postnatal retina

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**Abstract:** Epidermal growth factor (EGF) signaling has been implicated in the regulation of the differentiation and proliferation of retinal progenitors. We assessed how different levels of EGF signaling, achieved either by increasing receptor expression or via addition of the exogenous ligand, or an increase in both, can affect the differentiation of progenitors in the first week of postnatal retinal development in the model system of retinal explants (REs). Proliferating progenitor cells in REs were infected with either the control CLV3/ESR-related peptide family (CLE)-green fluorescent protein (GFP)- or with EGF receptor (EGFR)-GFP-expressing retrovirus, and grown in the control medium or in the presence of exogenous EGF (10 ng/mL). The differentiation of infected cells into Müller glia (Sox9<sup>+</sup>), rod photoreceptors (rhodopsin<sup>+</sup>) and horizontal cells (calbindin<sup>+</sup>) was analyzed. In all the examined conditions, infected cells differentiated into Müller glia and rod photoreceptors that normally develop postnatally. Horizontal cells finished their development during the embryonic stages and progenitors infected with control-GFP virus did not differentiate into GFP<sup>+</sup>/calbindin<sup>-</sup> in either control or EGF-supplemented medium, however, cells infected with EGFR-GFP differentiated into horizontal cells (GFP<sup>+</sup>/calbindin<sup>+</sup>) in both culture conditions. These results imply that altering the levels of EGFR and/or the amount of the EGF ligand can overcome progenitor competence restriction.

**Keywords:** retina; EGFR; progenitors; differentiation; postnatal development

### INTRODUCTION

The vertebrate neural retina is comprised of six types of neurons: rod and cone photoreceptors, amacrine cells, retinal ganglion cells (RGCs), horizontal cells, bipolar cells and one type of glia, Müller glia. These cell types are all derived from one population of proliferating multipotent progenitors [1] during embryonic and postnatal development through sequential and tightly regulated differentiation steps involving both cell intrinsic and extrinsic factors [2]. The seemingly homogenous populations of progenitor cells differ in their ability to respond to gradients of multiple extracellular signals present in the developing tissue.

Epidermal growth factor (EGF) signaling is one of the signaling pathways that regulate retinal development. Different cellular response to EGF can be

achieved either by a different concentration of ligand or with graded activation of receptors, accomplished by regulating ligand levels, as in the case of *Drosophila* [3-5] and *Caenorhabditis elegans* [6]. Furthermore, progenitor cell competence to respond to specific environmental signals may be either lost or acquired during development. The expression of the EGFR in developing retina starts during embryonic development between embryonic day 15 (E15) and E18 [7], reaching its peak at P4 and then declining [8]. Different combinations of various factor concentrations of the ligand and different levels of receptor expression can produce different levels of EGF signaling at developmental stages, eliciting distinct outcomes. Studies on PC12 cells indicated that lower levels of EGF-receptor (EGFR) expression and activation resulted in proliferation, while higher levels resulted in ligand-dependent

neuronal differentiation [9]. EGF in postnatal retinal development has thus far been closely investigated as a proliferation and differentiation factor for Müller glia [8,10]. Nevertheless, several lines of investigation have revealed that EGFR signaling has a nonmitotic function and is involved in cell fate decisions [10-12]. Studies on retinal development showed that the introduction of additional EGFR into progenitor cells in retinal explant cultures did not enhance proliferation but induced an increase in the proportion of clones that contain Müller glial cells [10]. These results suggest that receptor level is usually the limiting factor.

Different manipulations of the level of receptor expression *in vivo* in the brain and retina showed that if the critical level of active receptor is present on the cell surface it can allow sustained activation of intracellular signal transduction and change the properties and potential of such a progenitor cell [10,13-15]. These findings demonstrate that responsiveness to extracellular signals during development can be modulated by the introduction of additional receptors and/or ligands and suggest that the level of expression of receptors for these signals contributes to the regulation of cell fate.

It is unclear how restriction is the specific developmental stage that affects the competence potential of progenitor cells and whether competence can be altered by modifications of the levels of EGF signaling. Higher levels of EGFR-mediated signaling alone do not specify glial fate, indicating that the competence to generate glia is temporally regulated by additional mechanisms [10]. In the present study, we analyzed how altering the levels of EGF signaling with the addition of extra EGFR via retroviral infection or by addition of exogenous EGF ligand or both, affects cell fate choice of P0 postnatal retinal progenitors. By reducing the discrepancy in EGFR expression among early and late progenitor cells, additional changes in progenitor cells that regulate their competence to generate glia were revealed [14]. We showed that altered levels of EGF signaling enabled postnatal proliferating progenitors to develop into neuronal cell types whose differentiation

is completed during embryonic development, and that they do not normally differentiate postnatally. These data indicate that modifying the levels of EGF signaling can change the competence of postnatal retinal progenitors.

## MATERIALS AND METHODS

### Ethics statement

Animal experiments were approved by the Animal Ethics Committee of Instituto de Medicina Molecular (AEC\_027\_2010\_DH\_Rdt\_general\_IMM) (Lisbon, Portugal) and according to National Regulations.

### Retrovirus production

EGFR-GFP and CLE-GFP plasmids were generated by Sun et al. [16] from the original pCLE retroviral vector [17] and were a kind gift from Dr. Sally Temple (University at Albany, State University of New York, Albany, NY). Replication-deficient viruses with vsv-G coats were generated from these constructs as described previously [18]. Briefly, viral titers were determined in colony-forming units (CFUs) by incubating C6 glioma cells with serial dilutions of retrovirus in 10 steps. At 48 h post-infection, the number of GFP<sup>+</sup> cell clusters was counted. The CFUs were calculated by multiplying the number of GFP<sup>+</sup> cell clusters by the dilution factor. The titer of both the CLE-GFP and EGFR-GFP viruses was 10<sup>6</sup>/μL. Viruses were added to explants immediately after culturing.

**Table 1.** Schematic presentation of the differentiation potential of retinal progenitors in 7-DIV REs in different experimental paradigms. P0 progenitors infected with control-GFP and EGFR-GFP retrovirus differentiate into Müller glia and rod photoreceptors in either control or EGF-supplemented medium. P0 progenitors infected with EGFR-GFP and grown in either control or EGF-supplemented medium differentiate into horizontal cells, while the progenitors infected with control-GFP do not.

	Horizontal cells	Müller glia	Rod photoreceptors
<b>Retrovirus</b>	Calbindin <sup>+</sup> /GFP <sup>+</sup>	Sox9 <sup>+</sup> /GFP <sup>+</sup>	Rhodopsin <sup>+</sup> /GFP <sup>+</sup>
Control-GFP	–	+	+
Control-GFP+EGF 10 ng/mL	–	+	+
EGFR-GFP	+	+	+
EGFR-GFP+EGF 10 ng/mL	+	+	+

EGFR – epidermal growth factor receptor, GFP – green fluorescent protein. EGF – epidermal growth factor.

## Mouse strain and sample collection

C57BL/6 mice were fed *ad libitum* and housed in specific-pathogen-free (SPF) facilities. For the production of P0 pups, three female mice were housed with one male mouse and the date of the vaginal plug formation was established in order to ensure the precise timing of the birth. At birth (P0), the eyes were enucleated (the eye balls were removed with curved forceps and the optic nerve was severed) from P0 animals (n=18) after decapitation and processed for culturing. Eyes used for the same experimental condition were never taken from the same animal.

## *In vitro* culture of retinal explants (REs)

The enucleated eyes were transferred to a Petri dish containing Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, USA) supplemented with 50 IU ( $\mu\text{g}$ )/mL penicillin-streptomycin (Invitrogen, Paisley, UK). Retinas were removed and cultured as described [19]. Briefly, the retinas were placed on membrane culture inserts (Millicell CM, Millipore, Bedford, MA, USA; pore size 0.4  $\mu\text{m}$ ) in 6-well plates (vitreous side down), and cultured in a culture medium modified from [19], in 50% minimal essential medium (MEM)-HEPES (Invitrogen, San Diego, CA, USA), 25% Hank's balanced salt solution (Invitrogen), 5.75 mg/mL glucose, 25 U/mL penicillin, 25 mg/mL streptomycin, 200 mM L-glutamine, 1x Gibco B27 (Thermo Fischer Scientific, USA) and 1x Gibco N2 (Thermo Fischer Scientific)).

## Treatments of retinal explants

EGF-treated explants were cultured for 7 days *in vitro* (DIV) in medium containing 10 ng/mL EGF (Sigma), and the medium was changed every other day. Control explants were cultured in normal culture medium. At 7 DIV, the REs were fixed in 4% paraformaldehyde (PFA) and processed for immunofluorescence (IF).

## Retroviral infection

After the retinas were placed in a culture dish, 5  $\mu\text{L}$  of either the control CLE-GFP or EGFR-GFP-expressing retrovirus was added in drops on the upper surface of the explants. The explants were left on 37°C and

the media was changed as described. Two-4 different retroviral infections were performed for each experimental paradigm analyzed.

## Immunofluorescence

Eyes and retinal explants were fixed in 4% paraformaldehyde at 4°C overnight, cryoprotected in 30% sucrose and embedded in 7.5% gelatin; 15% sucrose and 12  $\mu\text{m}$  sections were used in the analysis. For IF, sections were degelatinized at 37°C for 15 min in 1x phosphate buffer solution (PBS) and permeabilized using Triton X-100 (0.5%) for 15 min. This was followed by blocking (10% normal goat serum, 0.1% TritonX-100) for 1 h at room temperature (RT) The primary antibodies used in this study were as follows: mouse anti-transcription factor Sox9 (1:500, DSHB), mouse anti-calbindin (1:500, Sigma), mouse anti-rhodopsin (1:500, DSHB) and chicken anti-GFP (1:1000, Abcam). The sections were incubated in the primary antibodies overnight at 4°C, washed and incubated with appropriate Alexa Fluor (488 or 594) conjugated secondary antibodies (1:400, Molecular Probes) for 1 h at RT.

## Imaging and analysis

Images were obtained with a Leica DM5000B microscope (Leica, Wetzlar, Germany). The findings reported as microscopic images were representative of observations performed in two-to-four REs.

## RESULTS

### Retroviral infection and culturing of retinal explants

In order to investigate how different levels of EGF signaling affect postnatal retinal cell specification, we used an *ex vivo* model system of REs [19] (Supplementary Fig. S1A). We wanted to analyze whether the addition of exogenous EGF affects the organization and cell specification in postnatal REs. Different levels of EGF signaling can render different outcomes in cell type specification and differentiation. As the concentration of the receptor was shown to be the limiting factor in EGF signaling [10], we introduced EGFR into divid-

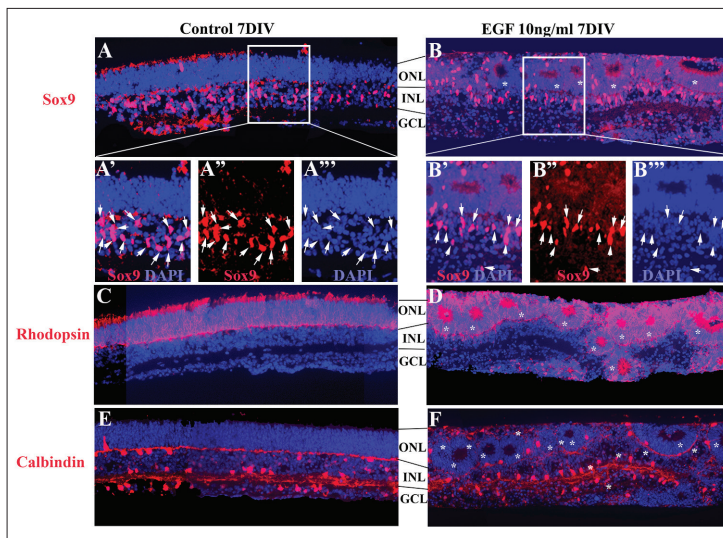


ing progenitors via the EGFR-GFP retrovirus (Supplementary Fig. S1A, B) immediately after culturing. To understand how different levels of EGF signaling affect the development of specific cell types in postnatal retina, we cultured P0 REs under four different conditions: (i) progenitors infected with control-GFP and grown in the control media; (ii) progenitors infected with control-GFP and grown in the presence of the exogenous EGF (10 ng/mL); (iii) progenitors infected with EGFR-GFP and grown in the control medium; (iv) progenitors infected with EGFR-GFP and grown in the presence of exogenous EGF (10 ng/mL) (the schematic overview of the experimental paradigm is presented in Supplementary Fig. S1A). The continuous presence of the receptor in conditions with and without added EGF provided graded levels of EGF signaling and established the experimental paradigm in which we could compare the outcomes of the increasing levels of EGF signaling. Retroviral infection allowed us to follow the destiny of the infected progenitors (GFP<sup>+</sup> cells) at 7 DIV (Supplementary Fig. S1C).

### The addition of the exogenous EGF did not alter the development of Müller glia, rod photoreceptors and horizontal cells

REs were isolated from P0 eyes and cultured either in the control medium (Fig. 1A, A', A'', C, E), or in the presence of exogenous EGF (10 ng/mL) (Fig. 1B, B', B'', D, F). After 7 days, the REs were analyzed for the expression of cell type specific markers such as Sox9, the marker for Müller glia, calbindin, the marker for horizontal cells and rhodopsin, the marker for rod photoreceptors. Horizontal cells appear during embryonic development, and rods and Müller glia are the cell types that appear postnatally [20]. The presence of EGF led to the formation of rosettes in the outer nuclear layer (ONL) (Fig. 1B, D, E, asterisk) but the general pattern of expression was preserved for all the markers analyzed. In control explant nuclei of Sox9<sup>+</sup>, the cells were distributed in the inner nuclear layer (INL), forming a ribbon bordering the basal part of the outer nuclear layer (ONL) (Fig. 1A, A', A''). A similar pattern was observed in the EGF-treated REs

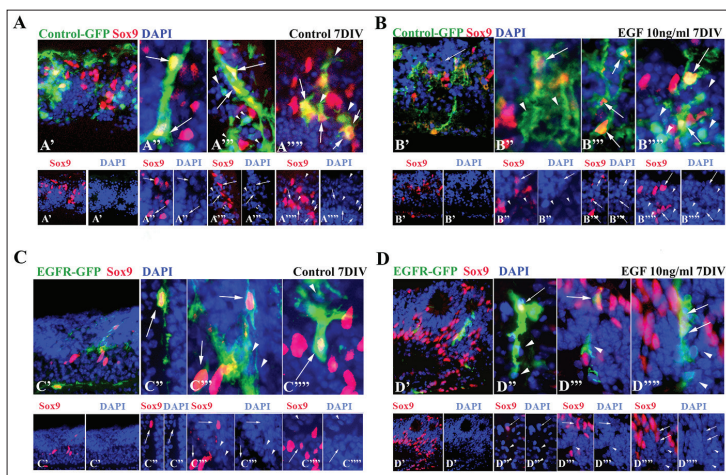
where the Sox9<sup>+</sup> cell distribution followed the rosette organization marking the basal border of the ONL (Fig. 1B, B', B''). In the control REs, rhodopsin staining labeled the rod photoreceptors in the ONL (Fig. 1C). Similarly, in EGF-treated REs, rhodopsin staining delineated the ONL organized in rosettes (Fig. 1D). calbindin<sup>+</sup> cells were distributed throughout the INL, with nuclei located in the apical and basal border of the INL in control REs (Fig. 1E). In the EGF-treated REs, a similar pattern of calbindin<sup>+</sup> cells assuming a rosette organization was present as well (Fig. 1F).



**Fig. 1.** The addition of exogenous EGF did not alter the development of Müller glia, rod photoreceptors and horizontal cells. Micrographs of 7DIV REs showing the organization and general pattern of expression of Müller glia (Sox9<sup>+</sup>, red) (A, B), rod photoreceptors (rhodopsin<sup>+</sup>, red) (C, D) and horizontal cells (calbindin<sup>+</sup>, red) (E, F) in control and EGF-treated REs. The patterns of expression of Sox9 (D, E), rhodopsin (F, G) and calbindin (H, I) were not altered in the EGF-treated REs (E, G, I) when compared to control REs (D, F, H). All sections are stained with DAPI (blue) to label the nuclei. For the Sox9 staining micrographs, higher magnification images were added to confirm nuclear expression of Sox9 (A', A'', A''', B', B'', B'''). Arrows point to Sox9 expression in the nuclei. \* – labeled rosettes. These are representative images from 2-4 REs analyzed. ONL – outer nuclear layer, INL – inner nuclear layer, GCL – ganglion cell layer. EGF – epidermal growth factor.

### P0 progenitors differentiate into Müller glia in the presence of different levels of EGF signaling

Müller glia are the only glial cell type in the retina and develop primarily during the first postnatal week. It has been shown that premature Müller cell differentiation is normally achieved by increased expression of EGFR and/or exposure to high local concentrations of ligand [10]. We analyzed



**Fig. 2.** P0 progenitors differentiate into Müller glia in the presence of different levels of EGF signaling. Micrographs of 7-DIV REs infected with control-GFP (A, B) or EGFR-GFP (C, D) retrovirus were analyzed for the expression of GFP (green) in Müller glia (Sox9<sup>+</sup>, red) in different experimental conditions. A – REs infected with control-GFP virus and grown in control media. B – REs infected with control-GFP and grown in the presence of EGF. C – REs infected with EGFR-GFP and grown in control media. D – REs infected with EGFR-GFP and grown in the presence of EGF. Arrows indicate double-labeled GFP<sup>+</sup>/Sox9<sup>+</sup> cells. Arrowheads indicate GFP<sup>+</sup>/Sox9<sup>-</sup> cells. The results are presented as lower magnification (20x) micrographs (A, B, C, D) and higher magnification micrographs with only few cells visible (all other micrographs). Below each micrograph there are corresponding micrographs representing separate channels (red, for the specific marker in question and blue for DAPI nuclear staining). These are representative images from 2–4 REs analyzed. GFP – green fluorescent protein, EGF – epidermal growth factor, EGFR – epidermal growth factor receptor.

if the virus-infected cells differentiate into Müller glia in all the conditions examined. Double-labeling with anti-Sox9 and anti-GFP antibodies revealed double-labeled cells (Fig. 2, arrows) in all conditions. Progenitors infected with the control virus developed into Sox9<sup>+</sup> cells (Müller glia) in the absence or presence of exogenous EGF (Fig. 2A, B, arrows). Similarly, progenitors infected with the EGFR-GFP virus differentiated into Müller glia in both control media and in the presence of exogenous EGF (Fig. 2C, D). However, in REs infected with the control virus, some infected cells (GFP<sup>+</sup>) were Sox9 negative (-), indicating that infected progenitors were capable of differentiating into other cell types in the presence or absence of EGF (Fig. 2A, B, arrowheads). Similarly, some progenitors infected with the EGFR-GFP virus and grown with or without EGF were Sox9<sup>-</sup>, indicating that these cells can adopt fates other than Müller glia even with continuous EGFR expression (Fig. 2C, D, arrowheads). Thus, different levels of EGF signaling did not inhibit control and EGFR-infected progenitor cells from dif-

ferentiating into Müller glia during the first postnatal week in retinal development, but did not exclusively drive progenitors into the glial fate.

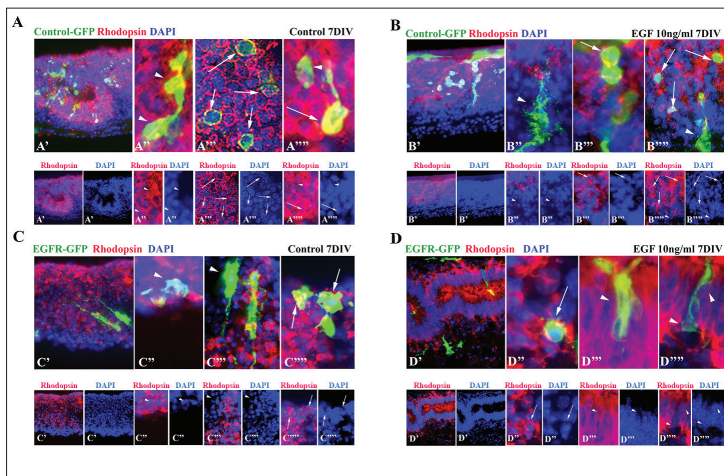
### P0 progenitors differentiate into rod photoreceptors in the presence of different levels of EGF signaling

The other cell types whose differentiation peaks during the first postnatal week are rod photoreceptors. The normal fate of P0 progenitor cells in rat retina *in vivo* is to develop predominantly into rod photoreceptors [21]. We examined whether P0 progenitor differentiation in rods (rhodopsin<sup>+</sup>) is affected by different levels of EGF signaling. Double-labeling with anti-rhodopsin and anti-GFP antibodies revealed the presence of double-labeled cells (Fig. 3, arrows) in all of the conditions. Progenitors infected with the control virus developed into rhodopsin<sup>+</sup> cells (rod photoreceptors) in the absence and in the presence of exogenous EGF (Fig. 3A, B, arrows). Similarly, progenitors infected with the EGFR-GFP virus differentiated into rhodopsin<sup>+</sup>/GFP<sup>+</sup> cells in both control media and in the presence of exogenous EGF (Fig. 3C, D). In all the conditions examined, the bulk of progenitors, infected with either control or EGFR-GFP virus, developed into rhodopsin<sup>-</sup> cells (arrowheads). Thus, different levels of EGF signaling did not inhibit the expression of rhodopsin in the infected, GFP<sup>+</sup> cells during the first postnatal week of retinal development.

### Increased levels of EGF signaling permit postnatal differentiation of progenitors into horizontal cells

Horizontal cells are retinal interneurons that appear early on, from embryonic day 11 (E11), and they finish their differentiation by E18. We examined whether different levels of EGF signaling affected the development of cell types whose differentiation process was finalized before birth, during embryonic development. We sought to determine how different experimental paradigms affect the differentiation of infected progenitors into horizontal cells (calbindin<sup>+</sup>) [22] in all





**Fig. 3.** P0 progenitors differentiate into rod photoreceptors in the presence of different levels of EGF signaling. Micrographs of 7DIV REs infected with control-GFP (A, B) or EGFR-GFP (C, D) retrovirus were analyzed for the expression of GFP (green) in rod photoreceptors (Rhodopsin<sup>+</sup>, red) in different experimental conditions. A – REs infected with control-GFP virus and grown in control media. B – REs infected with control-GFP and grown in the presence of EGF. C – REs infected with EGFR-GFP and grown in control media. D – REs infected with EGFR-GFP and grown in the presence of EGF. Arrows indicate double-labeled GFP<sup>+</sup>/rhodopsin<sup>+</sup> cells. Arrowheads indicate GFP<sup>+</sup>/rhodopsin<sup>-</sup> cells. The results are presented as lower magnification (20x) micrographs (A', B', C', D') and higher magnification micrographs with only few cells visible (all other micrographs). Below each micrograph there are corresponding micrographs, representing separated channels (red, for the specific marker in question, and blue for DAPI nuclear staining). These are representative images from 2-4 REs analyzed. GFP-green fluorescent protein, EGF – epidermal growth factor, EGFR – epidermal growth factor receptor.

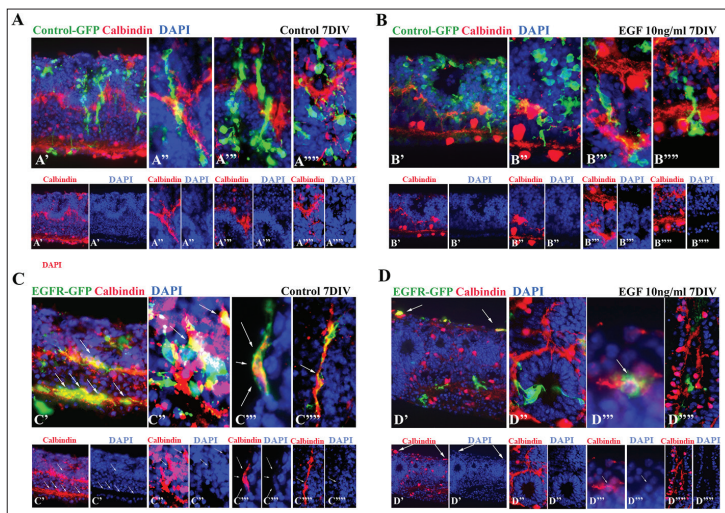
analyzed experimental paradigms. Double-labeling with anti-calbindin and anti-GFP antibodies revealed no double-labeled cells in REs infected with control virus, with or without the addition of the exogenous EGF (Fig. 4A, B), as was expected. However, a proportion of P0 progenitors infected with EGFR-GFP virus were calbindin<sup>+</sup>/GFP<sup>+</sup> in REs grown in control medium (Fig. 4C; C', C'', C''', arrows). Similarly, REs infected with EGFR-GFP and grown with the addition of the exogenous EGF resulted in the presence of double-labeled, calbindin<sup>+</sup>/GFP<sup>+</sup>, cells (Fig. 4D'', arrows). Therefore, the continuous expression of EGFR in progenitor cells, either in control media or in the presence of exogenous EGF, permitted for the differentiation of horizontal cells in postnatal REs.

## DISCUSSION

The major signaling pathways and their molecular constituents are evolutionarily highly conserved.

The morphogen, any diffusible signaling molecule, can, upon reaching the receiving cell, induce multiple different cell fates depending on its levels of expression. On the other hand, a number of receptors in the receiving cell can regulate different signaling thresholds and consequently, different differentiation outcomes. Therefore, the quantitative nature of signaling has raised a variety of questions [23-27]. Similarly, the quantitative nature of EGF signaling in development has been addressed in several studies and in different experimental models [28-29]. The introduction of extra EGFR *in vivo* increased the proportion of clones that contained Müller glial cells suggesting that receptor levels are normally limited [10]. The introduction of extra EGFR into ventricular zone (VZ) cells in the brain via retroviral infection resulted in premature expression of features characteristic of late SVZ progenitors [13]. Similarly, the introduction of EGFR via retroviral infection into oligodendrocyte progenitors hindered their final differentiation and extended their migratory behavior [15]. Activation of EGFR in retinal precursors can regulate proliferation and differentiation *in vitro* and *in vivo* [7,8,10,14,30,31]. These studies indicate that the level of EGFRs expressed by progenitor cells in the brain cortex and retina can contribute to the timing of their maturation and choice of response to pleiotropic environmental signals.

Retinal progenitor proliferation peaks around the day of birth and declines until about the end of the first postnatal week [20,32]. Cell division in postnatal retina ceases by P5-P6 days in the center of the retina, and by P11 in the periphery. Among cells produced postnatally, 73% differentiate as rods, 20% as bipolar cells, 6% as Müller cells and 1% as amacrine and ganglion cells [20]. In our study, P0 progenitors were infected with control and EGFR virus and grown either in control media or media supplemented with exogenous EGF (10 ng/mL), forming experimental groups with different levels of EGF signaling. This concentration was chosen in order to avoid overstimulation of progenitor proliferation and to allow for biased differentiation, as reported previously [10,13]. The competence



**Fig. 4.** The increased levels of EGF signaling permit postnatal progenitor differentiation into horizontal cells. Micrographs of 7DIV REs infected with control-GFP (A, B) or EGFR-GFP (C, D) retrovirus were analyzed for the expression of GFP (green) in horizontal cells (calbindin<sup>+</sup>, red) in different experimental conditions. A – REs infected with control-GFP virus and grown in control media. B – REs infected with control-GFP and grown in the presence of EGF. C – REs infected with EGFR-GFP and grown in control media. D – REs infected with EGFR-GFP and grown in the presence of EGF. Arrows indicate double-labeled GFP<sup>+</sup>/calbindin<sup>+</sup> cells. The results are presented as lower magnification (20x) micrographs (A', B', C', D') and higher magnification micrographs with only few cells visible (all other micrographs). Below each micrograph there are corresponding micrographs, representing separated channels (red, for the specific marker in question, and blue for DAPI nuclear staining). These are representative images from 2-4 REs analyzed. GFP-green fluorescent protein, EGF – epidermal growth factor, EGFR – epidermal growth factor receptor.

of P0 progenitor cells to differentiate into Müller glia (Sox9<sup>+</sup>) and rod photoreceptors (rhodopsin<sup>+</sup>) cells was not affected by different levels of EGF signaling. It is possible that the number of Sox9<sup>+</sup> and rhodopsin<sup>+</sup> cells was different between different conditions as a result of the effect that different levels of EGF signaling can have on proliferation. However, our study was focused on the influence of different levels of EGF signaling on progenitor competence, and consequently on their final differentiation outcome. Surprisingly, P0 progenitors infected with EGFR virus developed into horizontal cells (calbindin<sup>+</sup>) with or without the presence of exogenous EGF, while progenitors infected with the control virus did not. This suggested that the addition of extra receptor permitted the infected progenitors to differentiate into earlier fates that normally differentiate during the embryonic period, such as horizontal cells.

Horizontal cells, together with amacrine cells, lie within the INL and modulate signaling between pho-

toceptors and bipolar cells. They exhibit a variety of unique biological properties such as unusual migratory behavior, unique morphological plasticity and the ability to divide at a relatively late stage during development [33]. Finally, data indicating that fully differentiated horizontal cells can give rise to metastatic retinoblastoma challenge the assumption that tumors are derived solely from progenitor/stem-like cells [34]. It is possible that P0 progenitors with forced expression of EGFR regain the competence to differentiate into horizontal cells in the time frame generally not permissible for this cell fate choice. EGFR<sup>+</sup>/calbindin<sup>+</sup> cells present in REs grown without exogenous EGF indicated that the receptor is the limiting factor and that there is enough endogenous ligand in the explant to sustain the signaling, although it is possible that the addition of extra EGF affected the proliferation of EGFR<sup>+</sup>/calbindin<sup>+</sup> cells. As the horizontal cells have the ability to divide in a fully differentiated state [34], it is possible that under specific conditions of RE culturing, these cells entered the cycle and were thus capable of being infected with the retrovirus. However, this seems unlikely as we could not detect any GFP<sup>+</sup>/calbindin<sup>+</sup> cells in REs infected with the control virus in the presence of exogenous EGF. A more likely scenario is that some of the EGFR<sup>+</sup> P0 progenitors recapitulated the embryonic developmental program, resulting in the expression of the horizontal cell-specific marker.

The presence of continuous expression of EGFR in infected cells did not hinder the expression of the markers of final cell fates – Sox9, rhodopsin and calbindin. Moreover, the addition of the exogenous EGF also allowed for the expression of these markers, indicating that the increased level of EGF signaling is not sufficient to maintain these progenitors in the undifferentiated state. Several studies have suggested that progenitor cell competence to respond to specific environmental signals can either be lost or acquired during development. For example, competence to divide in response to TGF $\alpha$  was acquired between E15 and E18 [7], as was competence to respond to signals that promote rod development [35]. Thus, altering the

levels of EGF signaling cannot extend the time frame of progenitor competence indefinitely.

Horizontal cells form an extensive network that allows for the communication between photoreceptors and bipolar cells. The ablation of horizontal cells led to rod photoreceptor degeneration and induced extensive retinal network remodeling [36]. It is thus possible that in the case of photoreceptor degeneration, the ability to induce horizontal cell differentiation *de novo* and manipulate the levels of EGF signaling can facilitate the repair of retinal circuits or even enable photoreceptor regeneration. This is of particular importance because one of the main problems related to photoreceptor transplantation and regeneration is their inability to become incorporated into the existing retinal network and become fully functional.

The determination and differentiation of heterogeneous cell types within the context of complex tissues is the culmination of the expression of many gene products and their subsequent intra- and intercellular signaling events. Fully understanding the mechanisms underlying these processes is fundamental to many areas of biology. This knowledge will have widespread application in the treatments of developmental disorders and diseases, such as cancer, and will be critical for the successful bioengineering and transplantation of tissue types to replace damaged or degenerated structures.

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**Author contributions:** SI conducted the experimental research and presented the obtained results. She supervised all phases of the research and reviewed the manuscript. IJM and TA performed the literature search, prepared all the images and critically reviewed the manuscript. SK and DH supervised the research and the preparation of the manuscript. All authors read and approved the final manuscript.

**Conflict of interest disclosure:** The authors have no conflicts of interest to declare,

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### Supplementary Material

The Supplementary Material is available at: [http://serbiosoc.org.rs/NewUploads/Uploads/Ivkovic%20et%20al\\_4389\\_Supplementary%20Information.pdf](http://serbiosoc.org.rs/NewUploads/Uploads/Ivkovic%20et%20al_4389_Supplementary%20Information.pdf).