Smed-egfr-4 is required for planarian eye regeneration

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ABSTRACT Planarians are remarkable organisms that can regenerate their entire body from a tiny portion thereof. This capability is made possible by the persistence throughout the lifespan of these animals of a population of pluripotent stem cells known as neoblasts. Planarian neoblasts include both pluripotent stem cells and specialized lineage-committed progenitors that give rise to all mature cell types during regeneration and homeostatic cell turnover. However, little is known about the mechanisms that regulate neoblast differentiation. A recent study demonstrated that *Smed-egfr-1*, a homologue of the epidermal growth factor receptor (EGFR) family, is required for final differentiation, but not specification, of gut progenitor cells into mature cells. Given the expression by planarians of several EGFR homologues, it has been proposed that these homologues may have diverged functionally to regulate the differentiation of distinct cell types in these animals. In this study, we investigated the role of *Smed-egfr-4* in eye regeneration. Compared with controls, animals in which this gene was silenced by RNA interference (RNAi) regenerated smaller eyes. Moreover, the numbers of both mature eye cell types, photoreceptor neurons and cells of the pigment cup, were significantly reduced in *Smed-egfr-4(RNAi)* animals. By contrast, these animals exhibited an increase in the numbers of eye progenitor cells expressing the specific markers *Smed-ovo* and *Smed-sp6-9*. These results suggest that *Smed-egfr-4* is required not for the specification of eye progenitor cells but for their final differentiation, and support the view that in planarians the EGFR pathway might play a general role in regulating the differentiation of lineage-committed progenitors.

KEY WORDS: *planarian, regeneration, EGFR signalling, photoreceptor, stem cell differentiation*

Introduction

Unravelling the mechanisms that regulate stem cell differentiation is crucial in order to understand the amazing regenerative capabilities of freshwater planarians, in which a population of stem cells is maintained for the entire lifespan and can be activated in response to injury or amputation. This remarkable ability makes freshwater planarians a classical model for the study of stem cell-based regeneration (for recent reviews, see Cebrià *et al.*, 2018; Rink 2018). Planarian neoblasts are a heterogeneous cell population consisting of truly pluripotent undifferentiated stem cells, known as cNeoblasts (Wagner *et al.*, 2011; Zeng *et al.*, 2018), as well as specialized lineage-committed progenitors specific for distinct mature cell types (Scimone *et al.*, 2014; Zhu and Pearson, 2016). Although the cell-specific progenitors of most cell types have been largely characterized, relatively little is known about the genes and signalling pathways that regulate

the specification of these progenitors and their ultimate differentiation into mature cells (Zhu *et al.*, 2015; Solana *et al.*, 2013; Tu *et al.*, 2015). It has been recently proposed that the epidermal growth factor receptor (EGFR) signalling pathway may play an important general role in neoblast differentiation (Barberán and Cebrià, 2018). Silencing of *Smed-egfr-1* results in defects in planarian gut regeneration and maintenance (Barberán *et al.*, 2016a), reducing the number of newly differentiated mature gut cells and increasing the number of gut progenitors that fail to differentiate. *Smed-egfr-1* therefore appears necessary not for gut progenitor specification but for their ultimate differentiation (Barberán *et al.*, 2016a). In planarians, the EGFR family has undergone expansion and 6 homologues have been identified in the model species *Schmidtea mediterranea* (Barberán *et*

Abbreviations used in this paper: EGFR, epidermal growth factor receptor; RNAi, RNA interference.

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al., 2016b). It has been proposed that these different EGFRs, which show distinct expression patterns, participate in the differentiation of a variety of cell types, including neurons and protonephridia (Fraguas *et al.*, 2011; Rink *et al.*, 2011). Given the role of *Smed-egfr-1* in gut differentiation, the fact that distinct EGFR homologues are expressed in different tissues and cell types, and the implication of certain EGFRs in cell differentiation, one recently proposed model suggests that EGFRs play a general role in the final differentiation of different populations of lineage-committed progenitors (Barberán and Cebrià, 2018). In the present study, we investigated the role of *Smed-egfr-4* in eye regeneration. We found that silencing of *Smed-egfr-4* led to the formation of smaller eyes consisting of fewer photoreceptor neurons and pigment cup cells. Interestingly, this reduction in the number of mature eye cells correlated with an increase in the number of eye progenitors. These results suggest that *Smed-egfr-4* is required for the final differentiation of eye progenitor cells and support a general role of EGFR genes in planarian neoblast differentiation.

Results

Smed-egfr-4 is expressed ubiquitously, including in the eyes

To characterize the expression pattern of *Smed-egfr-4*, we performed *in situ* hybridization experiments in intact planarians. Whole-mount *in situ* hybridization revealed *Smed-egfr-4* expression in the cephalic ganglia, the pharynx, and the mesenchyme around the gut branches (Fig. 1A). Low levels of expression were also detected in photoreceptor cells (Fig. 1B). The expression pattern in the mesenchyme did not appear to correspond to neoblasts, since the *Smed-egfr-4* pattern was unchanged in animals in which the neoblast population was eliminated by irradiation (Fig. 1C). To evaluate *Smed-egfr-4* expression during regeneration, planarians were decapitated and the regeneration of a new head was monitored. *Smed-egfr-4* was expressed in the regenerative blastema from day 1 after amputation (Fig. 1D). From days 2–3, *Smed-egfr-4* was also detected in the newly formed brain primordia (Fig. 1 E–F) and high levels of expression were maintained within the blastema and in the regenerating cephalic ganglia throughout the entire regenerative process (Fig. 1 G–I).

Recently, two single-cell RNAseq atlas of *S. mediterranea* have been published (Fincher *et al.*, 2018; Plass *et al.*, 2018). Searches in them showed that *Smed-egfr-4* is expressed in a variety of tissues (in agreement with *in situ* hybridizations), including: neurons, muscle, gut, pharynx and epidermis (Fig. 1J). Also, *Smed-egfr-4* was found to be expressed in neoblasts (Fig. 1J). The fact that *Smed-egfr-4* seems to be expressed at low levels in neoblasts together with its higher expression in many other tissues could explain why its overall pattern after irradiation did not show obvious differences compared to that from non-irradiated animals (Fig. 4 A,C).

As the signal of *Smed-egfr-4* in the photoreceptors after *in situ* hybridiza-
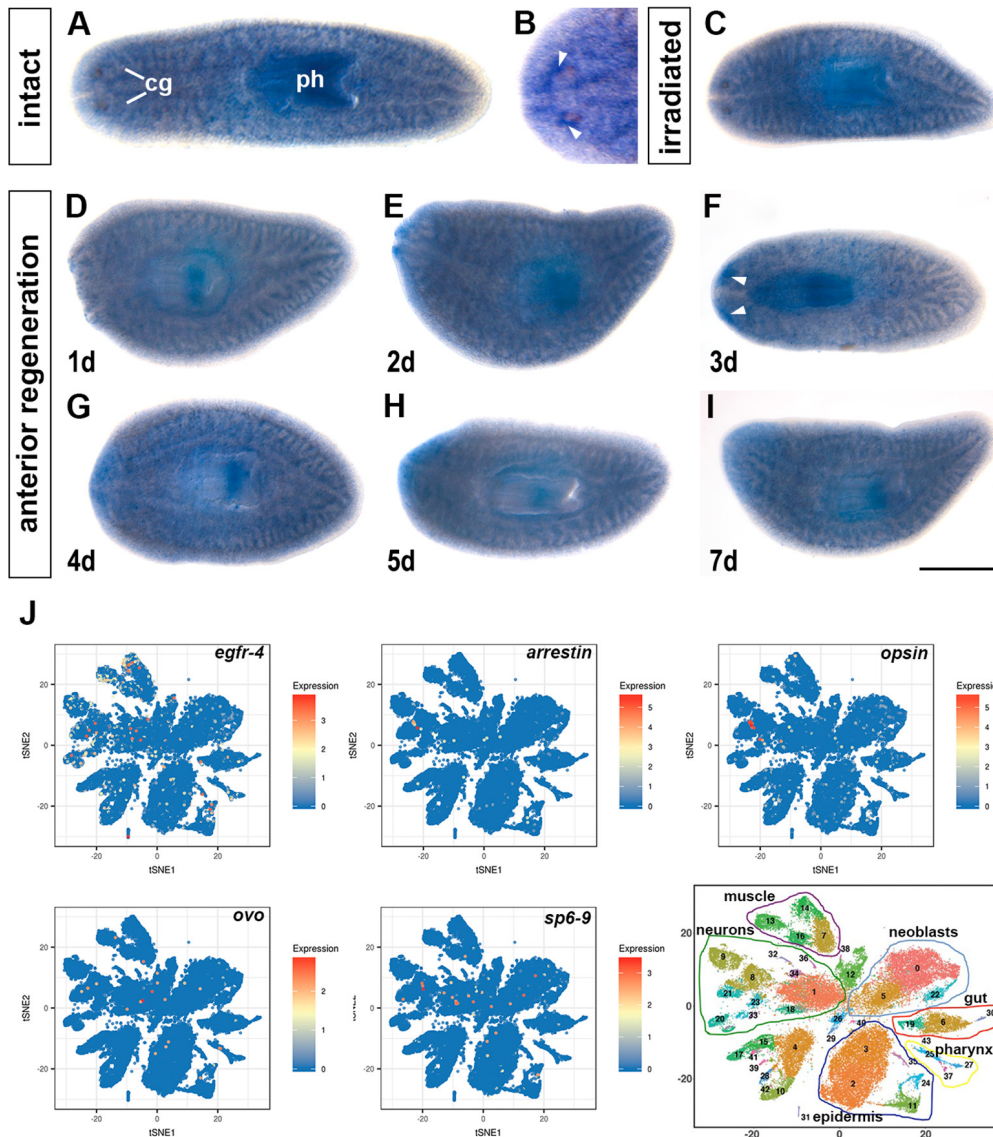
Fig. 1. ***Smed-egfr-4*** **expression in intact and regenerating planarians.** **(A,B)** Smed-egfr-4 *is expressed in the CNS, pharynx, and mesenchyme, and in photoreceptors (arrowheads in B).* **(C)** *Irradiation has no obvious effect on the pattern of* Smed-egfr-4 *expression.* **(D–I)** Smed-egfr-4 *expression during anterior regeneration. Arrowheads in* **(F)** *indicate the new brain primordia.* **(J)** *t-SNE plots for* egfr-4, arrestin *(dd_Smed_v6_17854_0_1; Brandl* et al., 2015), opsin *(dd_Smed_v6_15036_0_1; Brandl* et al., 2015), *ovo (dd_Smed_v6_48430_0_1; Brandl* et al., 2015) *and* sp6-9 *(dd_Smed_v6_17385_0_1; Brandl* et al., 2015) *obtained from http://digiworm.wi.mit.edu (Fincher* et al., 2018). *cg, cephalic ganglia; ph, pharynx. Scale bar, 400 μm.*

tions was faint we searched to compare its expression profile in the single-cell RNAseq atlas with that from other well-known genes expressed in the eyes such as arrestin, opsin, ovo and sp6-9 (Fig. 1J). Although no specific eye lineage has been identified in this single cell atlas, all these genes are expressed in cells within the neural cluster as *Smed-egfr-4* does. Therefore, if we combine the

expression data from *in situ* hybridizations and the single-cell atlas with the RNAi phenotype on eye regeneration described below, it seems quite probable that *Smed-egfr-4* is expressed in a variety of tissues including the photoreceptors.

Smed-egfr-4 silencing has no detectable effect on CNS regeneration

To characterize the function of *Smed-egfr-4*, we performed a series of functional RNAi experiments. Because *Smed-egfr-4* is expressed in the central nervous system (CNS), we first investigated whether its silencing resulted in defective regeneration of the cephalic ganglia. Whole-mount *in situ* hybridization for different neural-specific markers showed that *Smed-egfr-4(RNAi)* animals correctly regenerated the CNS, displaying normal patterns of expression of the different neuronal subpopulations analysed (Fig. 2), including GABAergic neurons (*Smed-gad, Nishimura et al., 2008*), dopaminergic neurons (*Smed-th, Nishimura et al., 2007*), and the brain lateral branches (*Smed-gpas, Cebrià et al., 2002*).

Smed-egfr-4 silencing results in the regeneration of smaller eyes

Planarian eyes consist of two distinct cell types, which can be
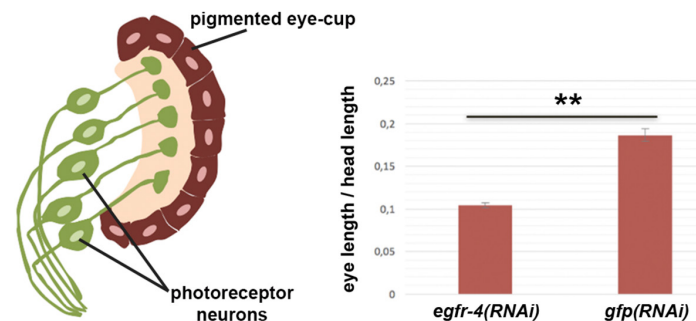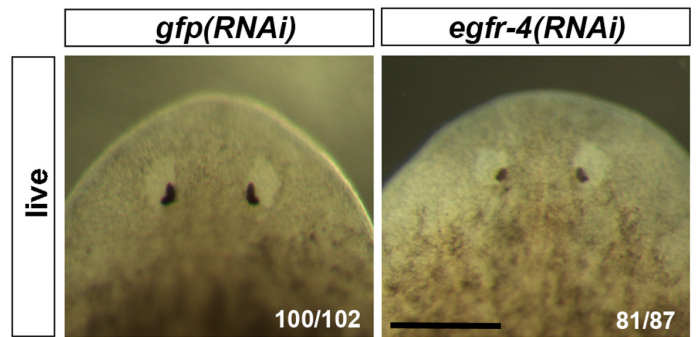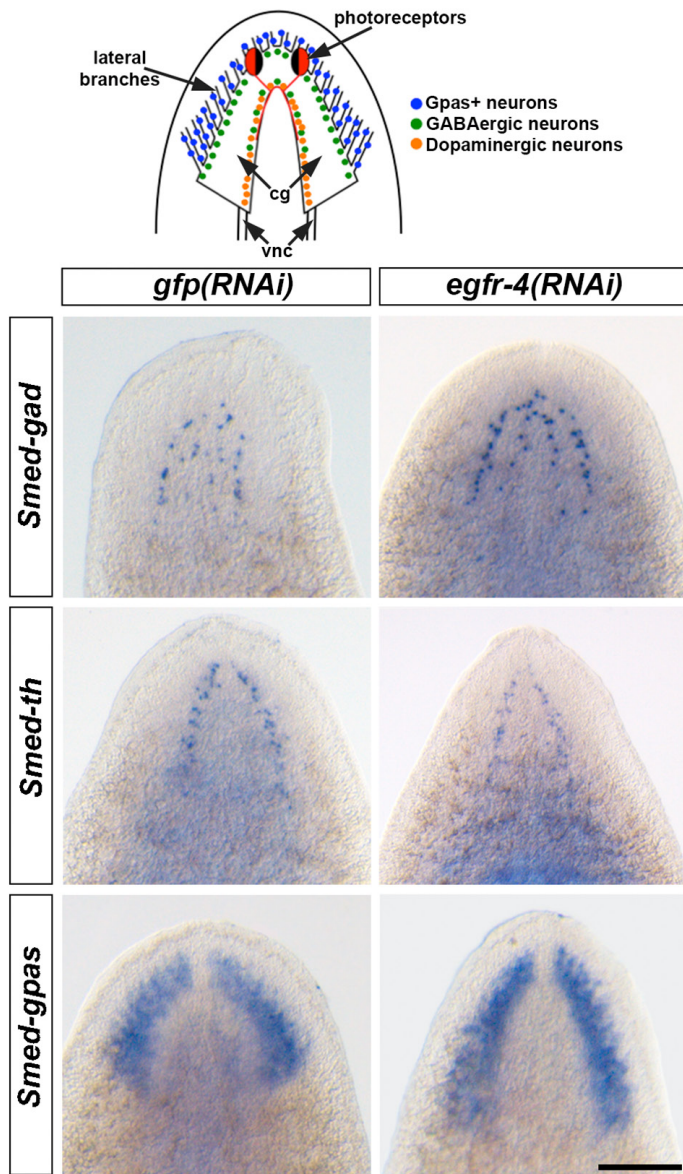
Fig. 2 (left). Normal CNS regeneration after *Smed-egfr-4 RNAi*. *In* Smed-egfr-4(RNAi) *planarians, the normal patterns of each of the different neuronal populations are re-established after 11 days of regeneration. The schematic drawing represents the cephalic ganglia with the relative positions of the neural markers used below* Smed-gad*: GABAergic neurons;* Smed-th*: dopaminergic neurons and* Smed-gpas*: G protein alpha subunit positive neurons.* Smed-gad *is expressed in neurons around the central neuropil with a higher density in the outer side of the cephalic ganglia.* Smed-th *is expressed in neurons mainly located along the medial side of the cephalic ganglia.* Smed-gpas *is expressed in neurons located in the cephalic lateral branches that project towards the head periphery. The photoreceptor neurons with their axonal projections are shown in red. cg, cephalic ganglia; vnc, ventral nerve cords. Scale bar, 150 μm.*

Fig. 3 (right). *Smed-egfr-4(RNAi)* planarians regenerate smaller eyes. Smed-egfr-4 *silencing results in the regeneration of smaller eyes during anterior regeneration. The schematic drawing shows the organization of the planarian eye with their two cell types: the pigmented eye-cup and the bipolar photoreceptor neurons that send rhabdomeric projections towards the pigmented eye-cup and axonal processes that project together into a specific brain region. The graph depicts the ratio of eye length to head length, measured from the base of the eye to the anterior tip of the head. n=40 eyes per treatment. **p<0.005 (Student's t-test). Error bars correspond to s.e.m. All samples correspond to 11 days of regeneration. Scale bar, 200 μm.*

easily distinguished in live animals: photoreceptor neurons and pigment eye-cup cells. Photoreceptor neurons are located in the whitish area adjacent to the eye pigment cup (Fig. 3). Given our observation that *Smed-egfr-4* is expressed in photoreceptor cells, we investigated the effects of a reduction in its expression on eye regeneration. Direct visual examination revealed that the eyes regenerated by *Sm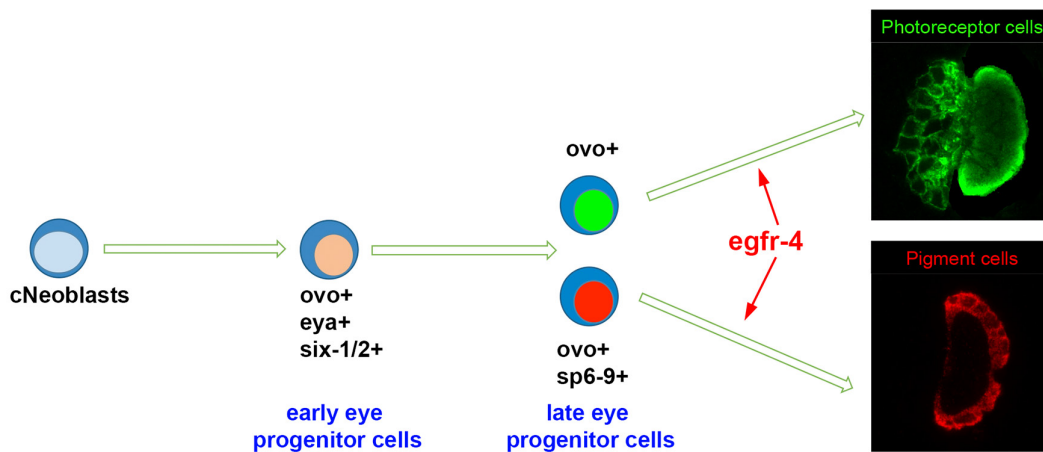ed-egfr-4(RNAi)* planarians were smaller than those of controls. To quantitatively characterize these apparent differences, and to account for the correlation between head and eye size (bigger heads have bigger eyes and smaller heads have smaller eyes), we measured the ratio of eye length to head length (measured from the posterior base of the eyes to the anterior most tip of the head). The measurements obtained confirmed that *Smed-egfr-4* silencing resulted in the regeneration of smaller eyes (Fig. 3).
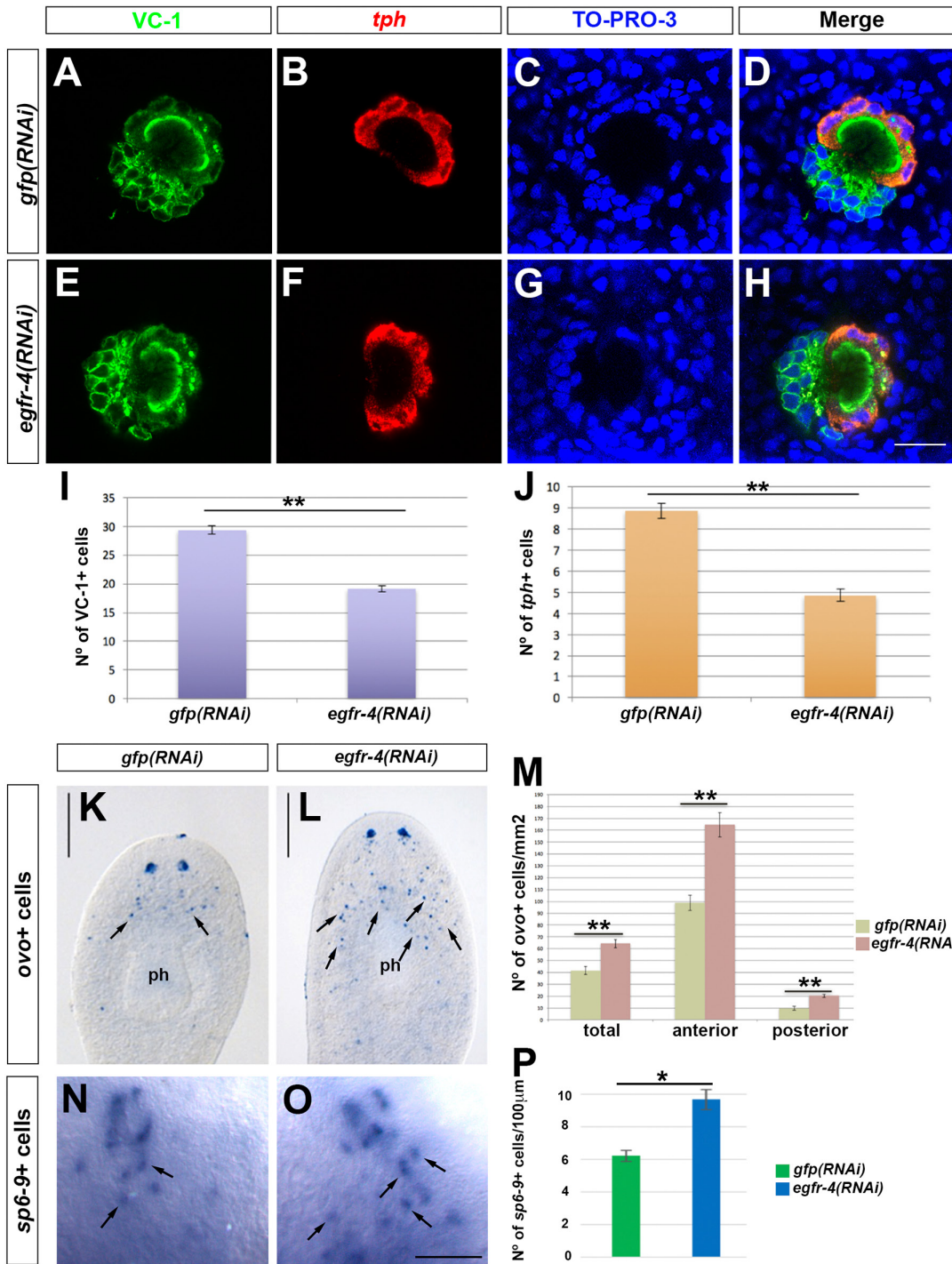
Fig. 4. *Smed-egfr-4 RNAi* results in a decrease in the number of mature eye cells and an increase in the number of eye progenitor cells. (A–H) *Double fluorescent* in situ *hybridization for* Smed-tph *and immunostaining with VC-1 to detect cells of the pigment cup (in red) and photoreceptor neurons (in green) in control gfp(RNAi)* **(A–D)**, *and* Smed-egfr-4 *(RNAi) animals* **(E–H)**. *Nuclear staining with TO-PRO-3 is shown in blue. Only Z-stacks of very few confocal planes are shown to clearly see the cellular organization and architecture of the eye. Scale bar, 20 μm.* **(I, J)** *Graphs depicting the numbers of VC-1+ and tph+ cells, respectively. In* **(I)**, *n=23 eyes per treatment. In* **(J)**, *n=15 eyes per treatment* **p<0.005 (Student's t-test). All samples correspond to 11 days of regeneration.* **(K,L)** *Whole-mount* in situ *hybridization for* Smed-ovo. *Arrows indicate eye progenitor cells in the pre-pharyngeal region. ph, pharynx. Scale bar, 300 μm.* **(M)** *Graph depicting the numbers of* ovo+ *cells throughout the organism.* Total *refers to the entire organism;* anterior *refers to the region between the tip of the head and the base of the pharynx;* posterior *refers to the region between the base of the pharynx and the posterior tip of the tail. All samples correspond to 10 days of regeneration. n=20 animals per treatment.* **p<0.005 (t-test).* **(N,O)** *Whole-mount* in situ *hybridization for* Smed-sp6-9. *Arrows indicate some eye progenitor cells in the pre-pharyngeal region. Scale bar, 40 μm.* **(P)** *Graph depicting the number of* sp6-9+ *cells in each condition. All samples correspond to 11 days of regeneration. n= 25 animals per treatment. *p<0.05 (Student's t-test). In all graphs error bars correspond to s.e.m.*

ovo+

cNeoblasts

ovo+
eya+
six-1/2+

**early eye
progenitor cells**

ovo+
sp6-9+

**late eye
progenitor cells**

egfr-4

Photoreceptor cells

Pigment cells

Fig. 5. Proposed model illustrating the role of *Smed-egfr-4* in eye regeneration. *See details within the main text.*

Smed-egfr-4(RNAi) animals regenerate fewer mature eye cells but more eye progenitors

Given that *Smed-egfr-4(RNAi)* planarians regenerated smaller eyes, we investigated whether this effect corresponded to a reduction in the number of eye cells. VC-1 is an anti-arrestin antibody that specifically recognizes planarian photoreceptor neurons (Sakai *et al.*, 2000). *Smed-tph* encodes a tryptophan hydroxylase homologue that is expressed in the cells of the eye pigment cup (Fraguas *et al.*, 2011). The cell bodies of photoreceptor neurons are clustered around the eye pigment cup. Thus, to quantify the number of photoreceptors and eye pigment cup cells we performed double *in situ* hybridization and immunostaining for *Smed-tph* and VC1, respectively (Fig. 4 A–H). Although the architecture of the eye and the organization of the photoreceptors and eye pigment cup cells was rather normal (Fig. 4 A-H), quantifications revealed a significant reduction in the numbers of both cell types after *Smed-egfr-4* RNAi (Fig. 4 I-J).

Smed-ovo labels is expressed in both mature and progenitor eye cells (Lapan and Reddien, 2012). Eye progenitor cells are distributed as two trails of cells located posterior to the mature eyes, and differentiate with increasing proximity to the mature eye (Lapan and Reddien, 2012). To determine whether the decrease in the number of mature eye cells induced by *Smed-egfr-4* silencing was caused by a reduction in the number of eye progenitors, we examined the effect of *Smed-egfr-4* RNAi on *Smed-ovo* expression. Remarkably, in contrast to the effect on mature cells, we observed an increase in the number of *Smed-ovo*+ cells in *Smed-egfr-4(RNAi)* planarians (Fig. 4 K–L). In control planarians, *Smed-ovo*+ cells were predominantly located between the mature eyes and the base of the pharynx. However, after *Smed-egfr-4* silencing, the distribution of *Smed-ovo*+ cells shifted to more posterior regions (Fig. 4 K–L). Quantification of the number of *Smed-ovo*+ cells revealed a significant increase in *Smed-egfr-4(RNAi)* animals (Fig. 4M). While *Smed-ovo* is expressed in progenitors of both photoreceptor neurons and pigment cup cells, *Smed-sp6-9* is selectively expressed in the progenitors of the pigment cup cells (Lapan and Reddien 2011, 2012). *Smed-egfr-4* silencing also resulted in a significant increase in the number of *Smed-sp6-9* cells (Fig. 4 N–P). Together, these results indicate that *Smed-egfr-4* does not contribute to the specification of pluripotent neoblasts into eye progenitors, but is most probably involved in regulating the final differentiation of these progenitors into mature eye cells.

Discussion

Recently, planarians are emerging as an excellent model in which to study stem cell differentiation *in vivo*. Planarian pluripotent stem cells give rise to lineage-committed progenitors, which in turn subsequently differentiate into all the cell types found in these animals (Scimone *et al.*, 2014; Zhu and Pearson, 2016). While little is known about how this differentiation process is regulated at the molecular level, several genes that participate in neoblast differentiation have been identified. For example, *Smed-mex-3* is required for specification of the different progenitor populations (Zhu *et al.*, 2015), while silencing of *Smed-not* impairs neoblast differentiation (Solana *et al.*, 2013).

Planarian eyes consist of two cell types: pigmented cells, which organize themselves to form the pigment eye-cup; and bipolar photoreceptor neurons, which send rhabdomeric dendrites to the cells of the eye pigment cup and visual axons to a medio-lateral region of the brain. Moreover, some visual axons project contralaterally crossing the midline and forming the optic chiasm (Sasaki *et al.*, 2000; Okamoto *et al.*, 2005). Recently, transcriptomic analysis of planarian eyes identified several genes that regulate the differentiation of pluripotent neoblasts to form eye progenitor cells and ultimately mature eye cells. The earliest progenitors committed to the eye lineage co-express *Smed-ovo*, *Smed-six-1/2*, and *Smed-eya*. Silencing of any of these genes inhibits eye regeneration (Pineda *et al.*, 2000; Mannini *et al.*, 2004; Lapan and Reddien 2012). It appears that both photoreceptor neurons and pigmented eye-cup cells are derived from a split in the lineage of these *ovo*+ early progenitors (Lapan and Reddien, 2012). Furthermore, not all photoreceptor neurons appear to be equal, as several subpopulations (anterior, dorsal posterior, and ventral posterior) can be distinguished based on their expression of specific markers (Collins *et al.*, 2010). Interestingly, *Smed-smad6/7-1* and *Smed-bmp* are involved in the differentiation of the anterior photoreceptor neurons (González-Sastre *et al.*, 2012).

In this study, we investigated the role of *Smed-egfr-4* in planarian regeneration. Despite its expression in the CNS, silencing of this gene had no effect on CNS regeneration, suggesting that other EGFRs may compensate for the lack of *Smed-egfr-4* function. However, we cannot rule out the possibility that *Smed-egfr-4* silencing gave rise to defects in the CNS that could not be detected with the markers we used. Our analysis revealed that *Smed-egfr-4* silencing

did result in significant decreases in both types of mature eye cells. Remarkably, this decrease was coupled with an increase in the number of *Smed-ovo*+ and *Smed-sp6-9+* progenitor cells located in the pre-pharyngeal region (Fig. 4). According to one current model of eye differentiation, pluripotent neoblasts (cNeoblasts) give rise to early eye progenitors that co-express *ovo*, *eya,* and *six-1/2* (Lapan and Reddien, 2012), and from which the two eye lineages are derived, subsequently giving rise to late eye progenitors. Those expressing *ovo*, *sp6-9,* and other factors give rise to the cells of the eye pigment cup, whereas those expressing *ovo* and other factors give rise to photoreceptor neurons (Lapan and Reddien, 2012). This posterior-to-anterior differentiation "gradient" might explain in part why after the silencing of *Smed-egfr-4* there is an increase of *ovo*+ cells that reach more posterior regions that the *sp6-9+* cells that also increase in number after the silencing of this gene, but remain in more relative anterior regions. The fact that *Smed-egfr-4* silencing interferes with the final differentiation of both lineages (photoreceptor neurons and cells of the eye pigment cup) and increases the numbers of *ovo*+ and *sp6-9+* cells suggests that *Smed-egfr-4* is required for the differentiation of both lineages following their divergence from a common early progenitor (Fig. 5). This putative autonomous role of *Smed-egfr-4* in eye progenitor differentiation is in line with that previously described for *Smed-egfr-1* during gut progenitor differentiation (Barberán *et al.*, 2016a), and supports the view that the EGFR signalling pathway in planarians might play a general role in the differentiation of lineage-committed progenitors (Barberán and Cebrià, 2018). Further studies will be required to identify the other factors involved in the final differentiation of late eye progenitors and to better characterize the contributions of other EGFRs to the differentiation of other planarian cell types.

Materials and Methods

Planarian culture

Asexual *S. mediterranea* from the BCN-10 clonal line were used in all experiments. Animals were fed with veal liver and starved for at least 1 week before all experiments. All experiments were done at 20°C.

RNA interference

RNA interference (RNAi) was performed as previously described (Sánchez Alvarado and Newmark, 1999). Control animals were injected with green fluorescent protein (GFP)-derived double-stranded RNA (dsRNA). All animals underwent two rounds of dsRNA injection and amputation, separated by 3–4 days.

In situ *hybridization and immunohistochemistry*

For whole-mount *in situ* hybridizations, animals were treated as previously described (King and Newmark, 2013). In order to visualize photoreceptor neurons, fluorescent *in situ* hybridization for *Smed-tph* was followed by immunostaining using the monoclonal antibody VC-1 (1:15,000) (Sakai *et al.*, 2000), and an Alexa 488-conjugated goat anti-mouse secondary antibody (1:400; Molecular Probes). TO-PRO-3 was used for nuclear staining. A Leica MZ16F stereomicroscope and a ProgRes C3 camera (Jenoptik) were used for analysis of non-fluorescent *in situ* hybridizations. Fluorescence images were captured using a Leica SPE confocal microscope. All images were processed and analysed using ImageJ and Photoshop.

Statistical analyses

Statistical analyses were performed using Microsoft Excel and R Software 3.5-0 (RStudio 1.1.453). All results are expressed as the mean e standard error of the mean (s.e.m.). Data were analysed by Student's t-test and a p-value <0.05 was considered statistically significant. All quantifications were performed by an observer blind to treatment conditions.

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