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Pten coordinates retinal neurogenesis by regulating Notch signaling

Hong Seok Jo, Kyung Hwa Kang, Cheol O. Joe and Jin Woo Kim

Corresponding author: Jin Woo Kim, KAIST

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 30 September 2011

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by two referees and their comments are provided below.

As you can see both referees appreciate the analysis. Referee #1 has relative minor concerns, while referee #2 has a number of different issues that should be resolved. Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of the referees. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS

Referee #1:

This is a very thorough and easy to read study addressing the role of Pten-Akt signaling during retinal neurogenesis. I have reviewed previous versions of this MS for another journal and am really pleased to see the improvements and editing. This paper will likely have a large impact for those studying neural development. The results, however, have implications beyond neurogenesis and will shape future studies on cancer and stem cell maintenance.

I only have minor concerns for the highly mechanistic study.

Specific Comments.

1. P6: "The increase in Pten-deficient cells in the Pten-cko retinas might have resulted from either hyperproliferation or enhanced cell survival, both of which are strongly supported by PI3K-Akt signaling pathway (reviewed in Cantley, 2002; Engelman et al, 2006; Salmena et al, 2008)."

This is an awkward statement - of course there is more Pten-deficient cells in Pten-cko retina! Do the author mean, "differentiated cells" (as marked by pax6-cre-GFP) instead of "Pten-deficient" ??

2. Fig 1C points to cells that are either Pten+/GFP- (arrows) or Pten+/GFP+. There is highlights the inefficiency of the cre (or stability of Pten). Clearly the authors provide a wealth of data that Pten is being knocked-out in their study - but this Figure and associated text makes things a bit confusing. Can the authors increase the magnification of this analysis, split the color channels and therefore provide a better estimate to the efficiency of recombination and knock-out of Pten? Maybe place this more detailed analysis in the Supplemental data.

3. It would be nice to include the pathway / model diagram of Sup Fig 6 to the main body (perhaps as Fig 6C). This nicely summarizes the major findings.

Referee #2:

I have reviewed a manuscript submitted by Jo et al., which is entitled "Pten coordinates retinal neurogenesis by regulating Notch signaling". The authors examined retinal phenotypes in the Pax6 alpha-Cre-mediated Pten-knockout mouse, Pten-cko. The Pten-cko retina consists of distal Pax6-expressing region and proximal Pax6-negative region, which correspond to Pten-deficient region and Pten-normal region, respectively. The authors found that Pten-deficient distal region expanded into the proximal region during development in the Pten-cko retina. The authors found that cell-cycle progression was faster and apoptosis was decreased in the Pten-deficient cells. Although cell proliferation was activated at the early stages such as E14.5 and P0, the fraction of proliferating progenitor cells drastically decreased due to premature neurogenesis at a later stage such as P4. The number of retinal neurons increased in the Pten-cko retina in the early stages of development, whereas the number of Muller glia cells decreased in the Pten-cko retina. From these observations, the authors concluded that neurogenesis is accelerated in Pten-deficient cells. Next, the authors found that Notch signaling is compromised in the Pten-cko retina. Ectopic introduction of Notch intracellular domain (NICD) rescued cell proliferation defects in the Pten-cko retina, suggesting that Notch signaling is involved in defects in the Pten-cko retinas. Overall, the manuscript is interesting. Quality of the data is also good. However, I have several concerns. Specific comments are shown below.

1. The authors found that Pten-cko; R26-lacZ-positive region expanded from distal to proximal region at E14.5 (Fig. 1D) and was occupied in the nearly entire neural retina at P4 (Fig. S1B). To elucidate whether this expansion is due to hyperproliferation or enhanced cell survival of Pten-cko cells, the authors examined TUNEL of the Pten-cko retina at P5 (Fig. S2). The authors showed that the fraction or number of TUNEL-positive cells among Pten-normal cells is higher than that of Pten-deficient cells, suggesting that Pten-deficient cells tend to survive or Pten-normal cells tend to undergo apoptosis in the Pten-cko retinas. Although these data may be significant, an earlier stage

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than P5 is more appropriate in order to examine the expansion process of the Pten-deficient cell region, because Pten-deficient cells are largely occupied in the entire neural retina at P4 (Fig. S1B). The authors should examine TUNEL in the interface area between lacZ-positive and lacZ-negative region in the Pten-cko; R26-lacZ retina at E14.5. Is TUNEL prominently observed in the lacZ-negative region? If this is the case, it is possible that this expansion of Pten-cko cells depend on cell competition mechanism?

2. Related to the comment (1), I guess that the authors indicated the percentage of the number of TUNEL positive cells to the total number of LacZ-negative cells (red column), LacZ-positive cells (yellow column) and both cells (blue column) in Figure S2B. Is it correct? Legend of Fig. S2B is not clearly described and confusing. The TUNEL-positive apoptotic cells (Is there TUNEL-negative apoptotic cells?) were counted and were shown as a graph. Blue column indicated the number of TUNEL cells. However, the term "TUNEL(+) cells (%)" was shown on Y axis in the graph. Which the number or the fraction of TUNEL cells is correct in Fig S2B?

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4. Fig. 3, 4 and 6: Related to the comment (3), the authors should compare the fraction of individual retinal cell types rather than their number (otherwise should normalize using the size of retinal area that the authors examined). The total cell number in the distal/proximal retina where the authors counted retinal cell-types may be higher in the Pten-cko retina than in the Pax6-aCre retina or may be different even in the Pten-cko retina depending on individual sections.

5. Fig. 4C: It is interesting that Pax6-aEGFP-positive cells coexpress Tuj1 in the Pten-cko retina, whereas Pax6-aEGFP-positive cells is Tuj1-negative in the Pax6-aCre retina. These observations suggest acceleration of neuronal differentiation in the Pten-cko retina. However, it is still unclear how neurogenesis is accelerated. It is possible that hyperproliferation causes premature neuronal differentiation even in the case that the percentage of the number of neurogenic cell division to the total cell division (fraction of neurogenic cell division in total cell division) is not increased in the Pten-cko retina. I suggest the authors to examine whether the fraction of neurogenic cell division and proliferative cell division are altered in the Pten;cko retina.

6. Fig. 5AB: The authors showed that the protein level of Hes1 is lower in the Pten-cko retina than in the Pax-aCre retina, and propose that NICD transcription activity is affected in the Pten:cko retina. It is possible that the translation or maintenance of Hes1 protein is compromised in the Pten:cko retina, thus I suggest the authors to examine mRNA level of Hes1 by quantitative PCR analysis.

7. Fig. S4: Notch1 and Dll1 expression in the most peripheral (distal) region of the neural retina seems to be weaker in the Pten-cko retina than in the Pax6-aCre retina. It is interesting to examine the expression of retinal stem cell makers such as Rax, c-myc. Is these gene expression expanded or retarded in the CMZ?

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9. The first paragraph in the discussion: The authors mentioned a similarity between mouse Pten-cko retina and Drosophila eye disc. Although PI3K-Akt signaling is involved in neuronal differentiation in both tissues, it has not been shown whether PI3K-Akt signaling modulates Notch signaling pathway in Drosophila eye disc, and whether defects in the Pten-cko retina depend on insulin and

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11. The fourth paragraph in the discussion: the authors cited the paper suggesting that Notch signaling represses Pten expression (Palomero et al., 2007). This reference is missing in the reference list. Furthermore, the authors discussed on the possibility that a repeated cycle of Pten-mediated Notch activation and Notch-mediated Pten repression underlies the asymmetric generation of RPC and neurons. This discussion is too speculative, because a major input to activate Notch is probably its ligand Delta, which is omitted in Fig. S6.

12. In the discussion, the authors should discuss on how Akt activation inhibits Notch signaling, in particular, the interaction between NICD and other components, CBF1 and MAML1, by citing related references.

13. In the introduction (or discussion), the authors should mention previous literatures to report the roles of PTEN/PI3K-Akt in retina development, for example, Pimentel et al., (2002) Dev Biol. 247, 295-306; Kim et al. (2008) Gene and Dev 22, 3147-3157; Park et al. (2008) Science 322, 963-966.

1st Revision - authors' response 21 October 2011

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Specific Comments.

1. P6: "The increase in Pten-deficient cells in the Pten-cko retinas might have resulted from either hyperproliferation or enhanced cell survival, both of which are strongly supported by PI3K-Akt signaling pathway (reviewed in Cantley, 2002; Engelman et al, 2006; Salmena et al, 2008)." This is an awkward statement - of course there is more Pten-deficient cells in Pten-cko retina! ***Do the author mean, "differentiated cells" (as marked by pax6-cre-GFP) instead of "Pten-deficient" ??***

In here, 'Pten-deficient cells' means 'the R26-lacZ-positive cells that cover the entire Pten-cko retinas in contrast to the R26-lacZ-positive cells restricted only in the distal Pten-flox or Pax6-aCre retinas (Figure 1D and Figure S2)'. To avoid the confusion, we changed it as "The increase in the Pten-deficient cells, which are labeled by lacZ expression, in the Pten-cko retinas might have resulted from either hyperproliferation or enhanced cell survival, both of which are strongly supported by PI3K-Akt signaling pathway (reviewed in Cantley, 2002; Engelman et al, 2006; Salmena et al, 2008)." in the revised text (Page 6).

*2. Fig 1C points to cells that are either Pten+/GFP- (arrows) or Pten+/GFP+. There is highlights the inefficiency of the cre (or stability of Pten). Clearly the authors provide a wealth of data that Pten is being knocked-out in their study - but this Figure and associated text makes things a bit confusing. Can the authors increase the magnification of this analysis****, split the color channels and therefore provide a better estimate to the efficiency of recombination and knock-out of Pten?***

Maybe place this more detailed analysis in the Supplemental data.

We provide the split color channels in the revised Supplementary Figure 1.

3. It would be nice to include the pathway / model diagram of Sup Fig 6 to the main body (perhaps as Fig 6C). This nicely summarizes the major findings.

We moved the previous Supplementary Figure 6 to Figure 7 of the revised manuscript after some modifications.

Referee #2:

I have reviewed a manuscript submitted by Jo et al., which is entitled "Pten coordinates retinal neurogenesis by regulating Notch signaling". The authors examined retinal phenotypes in the Pax6 alpha-Cre-mediated Pten-knockout mouse, Pten-cko. The Pten-cko retina consists of distal Pax6-expressing region and proximal Pax6-negative region, which correspond to Pten-deficient region and Pten-normal region, respectively. The authors found that Pten-deficient distal region expanded into the proximal region during development in the Pten-cko retina. The authors found that cell-cycle progression was faster and apoptosis was decreased in the Pten-deficient cells. Although cell proliferation was activated at the early stages such as E14.5 and P0, the fraction of proliferating progenitor cells drastically decreased due to premature neurogenesis at a later stage such as P4. The number of retinal neurons increased in the Pten-cko retina in the early stages of development, whereas the number of Muller glia cells decreased in the Pten-cko retina. From these observations, the authors concluded that neurogenesis is accelerated in Pten-deficient cells. Next, the authors found that Notch signaling is compromised in the Pten-cko retina. Ectopic introduction of Notch intracellular domain (NICD) rescued cell proliferation defects in the Pten-cko retina, suggesting that Notch signaling is involved in defects in the Pten-cko retinas. ***Overall, the manuscript is interesting. Quality of the data is also good. However, I have several concerns. Specific comments are shown below.***

1. The authors found that Pten-cko; R26-lacZ-positive region expanded from distal to proximal region at E14.5 (Fig. 1D) and was occupied in the nearly entire neural retina at P4 (Fig. S1B). To elucidate whether this expansion is due to hyperproliferation or enhanced cell survival of Pten-cko cells, the authors examined TUNEL of the Pten-cko retina at P5 (Fig. S2). The authors showed that the fraction or number of TUNEL-positive cells among Pten-normal cells is higher than that of Pten-deficient cells, suggesting that Pten-deficient cells tend to survive or Pten-normal cells tend to undergo apoptosis in the Pten-cko retinas. Although these data may be significant, an earlier stage than P5 is more appropriate in order to examine the expansion process of the Pten-deficient cell region, because Pten-deficient cells are largely occupied in the entire neural retina at P4 (Fig. S1B). ***The authors should examine TUNEL in the interface area between lacZ-positive and lacZ-negative region in the Pten-cko; R26-lacZ retina at E14.5.*** *Is TUNEL prominently observed in the lacZ-negative region? If this is the case, it is possible that this expansion of Pten-cko cells depend on cell competition mechanism?*

We had also examined TUNEL-positive cells in embryonic retinas, but we found only few TUNEL-positive cells in both WT (*Pten-flox or Pax6-aCre*) and *Pten-cko* retinas. We have not included this in the body of the revised paper, due to space constraints, but have attached a file containing images of E14.5 TUNEL staining of the earlier stage retinal samples for Reviewer 2's inspection (Figures for referee not shown.) Based on very sparse TUNEL-positive cells in E14.5 *Pax6-aCre* and *Pten-cko* retinas, we conclude that distal-to-proximal expansion of *Pten*-deficient cells in E14.5 *Pten-cko* retinas is likely caused by hyperproliferation than enhanced survival of the *Pten*-deficient RPCs. However, relatively enhanced cell survival of *Pten*-deficient cells in comparison to wildtype neighbors during post-natal period might contribute to the predominance of *Pten*-deficient retinal cells in mature *Pten-cko* retina.

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2. Related to the comment (1), I guess that the authors indicated the percentage of the number of TUNEL positive cells to the total number of LacZ-negative cells (red column), LacZ-positive cells (yellow column) and both cells (blue column) in Figure S2B. Is it correct? Legend of Fig. S2B is not clearly described and confusing.

The red bar indicates average percentage of total TUNEL(+) cells regardless of their lacZ positivity. The blue bars mark the percentage of TUNEL(+)/lacZ(-) apoptotic WT cells, and the yellow bars represent the percentage of TUNEL(+)/lacZ(+) apoptotic Pax6-aCre lineage cells. We have corrected errors and also describe more clearly in the revised figure legend.

The TUNEL-positive apoptotic cells (Is there TUNEL-negative apoptotic cells?) were counted and were shown as a graph.

There could be apoptotic cells that cannot be detectable by TUNEL method. However, TUNEL assay is currently one of the most sensitive *in vivo* quantitative apoptotic assays. We have also detected apoptotic cells by staining active caspase-3, but we could not detect as many apoptotic cells as we detected by TUNEL staining method.

Blue column indicated the number of TUNEL cells. However, the term "TUNEL(+) cells (%)" was shown on Y axis in the graph. Which the number or the fraction of TUNEL cells is correct in Fig S2B?

As written above, the values in the Y-axis indicate the percentage of TUNEL-positive cells among DAPI(+) retinal cells. We explain the graph more clearly in the revised figure legend.

3. Fig. 2A-B: The authors showed the number of BrdU-positive cells per section. It is appropriate to show the fraction of BrdU-positive cells in distal retinal region (the percentage of the number of BrdU-positive cells to the total number of distal retinal region), because total cell number of distal retinal region is different depending on sections. Some normalization must be necessary for statistical analysis.

We have changed the Y-axis value to the percentage of the number of BrdU(+) cells (B) or Sox2(+) cells (D) to the total DAPI(+) cells of the area.

Furthermore, it is better to show the labeling of anti-pH3 antibody at least as supplementary figures.

We provide pH3 immunostaining images and their quantifications in Supplementary Figure 4.

4. Fig. 3, 4 and 6: Related to the comment (3), ***the authors should compare the fraction of individual retinal cell types rather than their number*** *(otherwise should normalize using the size of retinal area that the authors examined).*

We converted Y-axis values of the graphs to the percentage values (Figure 3M, 4B, 4D, 6B).

The total cell number in the distal/proximal retina where the authors counted retinal cell-types may be higher in the Pten-cko retina than in the Pax6-aCre retina or may be different even in the Pten-cko retina depending on individual sections.

To avoid misinterpretation of data, we always compare same area of retina along dorso-ventral and naso-temporal axes. Practically, we match coronal-sectioned slides that include nasal start point of optic disc head, and compare retinal sections locating same distance from the optic disc head margins.

5. Fig. 4C: It is interesting that Pax6-aEGFP-positive cells coexpress Tuj1 in the Pten-cko retina, whereas Pax6-aEGFP-positive cells is Tuj1-negative in the Pax6-aCre retina. These observations suggest acceleration of neuronal differentiation in the Pten-cko retina. However, it is still unclear

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how neurogenesis is accelerated. It is possible that hyperproliferation causes premature neuronal differentiation even in the case that the percentage of the number of neurogenic cell division to the total cell division (fraction of neurogenic cell division in total cell division) is not increased in the Pten-cko retina. ***I suggest the authors to examine whether the fraction of neurogenic cell division and proliferative cell division are altered in the Pten;cko retina.***

We determined proliferative and neurogenic cell division by measuring the division axes, i.e. horizontal (proliferative) vs. vertical (neurogenic), of RPCs in E14.5 *Pax6-aCre* and *Pten-cko* retinas. If division angle of DAPI-stained mitotic chromosomes is between 0^o and 45^o or 136^o and 180^o, it was classified as neurogenic division. Whereas, it is between 46^o and 135^o, we classified it as proliferative division. We could not find significant differences in the division axes of mitotic cells in *Pax6-aCre* and *Pten-cko* retinas. We provide the results for Reviewer 2's inspection (Figures for referee not shown.)

6. Fig. 5AB: The authors showed that the protein level of Hes1 is lower in the Pten-cko retina than in the Pax-aCre retina, and propose that NICD transcription activity is affected in the Pten:cko retina. ***It is possible that the translation or maintenance of Hes1 protein is compromised in the Pten:cko retina, thus I suggest the authors to examine mRNA level of Hes1 by quantitative PCR analysis.***

Since Cre-mediated deletion of *Pten* is not accomplished in the entire E14.5 mouse retinas (Figure 1D), it might be more accurate to compare the local amount of *Hes1* in Cre-affected distal retinas of *Pax6-aCre* and *Pten-cko* embryos. We added *in situ* hybridization results of *Hes1* in the revised Supplementary Figure 6B. The results show that *Hes1* in the distal part of E13.5 *Pten-cko* retina was significantly decreased in comparison to *Hes1* expression in equivalent area of littermate *Pax6-aCre* retina (Supplementary Figure 6B). We therefore concluded that remarkable decrease of Hes1 in *Pten-cko* retina was likely caused by defective *Hes1* transcription, which is regulated by NICD-CBF1-MAML1 complex (Fryer et al. (2002)).

7. Fig. S4: Notch1 and Dll1 expression in the most peripheral (distal) region of the neural retina seems to be weaker in the Pten-cko retina than in the Pax6-aCre retina. ***It is interesting to examine the expression of retinal stem cell makers such as Rax, c-myc. Is these gene expression expanded or retarded in the CMZ?***

We have not examined the expression of Rax and c-myc, but we have checked with other retinal stem cell markers, Sox9 and Pax6. We provide data that show the reduced expression of Pax6 and Sox9 in ciliary marginal zone (CMZ) of P7 *Pten-cko* retinas. Consequently, the numbers of cells in the CMZ of the post-natal *Pten-cko* retinas were less than those of WT littermates. We provide the results for Reviewer 2's inspection 3 and 4, respectively. (Figures for referees not shown.)

8. Fig. 6: The authors showed that expression of NICD suppressed the depletion of proliferating retinal cells in the Pten-cko retina at P4. However, the authors showed that the interaction between NICD and other components of Notch transcription complex such as CBP1 and MAML1 was weaker in the Pten:cko retina, resulting in the reduction of Hes1 transcription activity (Fig. 5D). ***This seems to be contradictory, because the NICD-mediated increase in pH3/Sox2 cell number (rescue efficiency for proliferation defects) is higher in the Pten-cko retina than in Pax6-aCre retina.*** *How do the authors explain these results?*

The contradictory effects of Akt on NICD transcription complex formation and cooperative stimulation of RPC proliferation with NICD could be interpreted in terms of stoichiometry of Akt to NICD in *Pten-cko* and *Pten-cko;R26-NICD* RPCs.
Endogenous Akt in *Pten-cko* retinal cells might be enough to influence negatively to endogenous NICD complex formation. However, the endogenous Akt might not be enough to cover ectopically overexpressed NICD in *Pten-cko;R26-NICD* retinal cells, although it can still affect to other endogenous cellular targets to stimulate cell proliferation. Therefore, ectopic NICD in *Pten-*

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cko;R26-NICD retinal cells could escape from Akt-induced inhibition and support RPC maintenance.

9. The first paragraph in the discussion: The authors mentioned a similarity between mouse Pten-cko retina and Drosophila eye disc. Although PI3K-Akt signaling is involved in neuronal differentiation in both tissues, it has not been shown whether PI3K-Akt signaling modulates Notch signaling pathway in Drosophila eye disc, and whether defects in the Pten-cko retina depend on insulin and mTOR signaling pathways. Thus, ***this discussion part is superficial, so it may be better to transfer it to the last paragraph.***

We move the paragraph to the second last part of Discussion in the revised manuscript (page 12 – 13).

10. The second paragraph in the discussion: The authors cited the previous report that hyperproliferation and premature depletion are observed in Pten-deficient HSCs and proposed that Pten plays a general role in preserving the stem cell population. However, in this study, the authors investigated only retinal progenitor cells but not retinal stem cells. ***It is important to examine whether retinal stem cells are affected in the Pten-cko retina (see the comment (7)).***

Please see our response to the Reviewer 2's comment (7).

11. The fourth paragraph in the discussion: the authors cited the paper suggesting that Notch signaling represses Pten expression (Palomero et al., 2007). ***This reference is missing in the reference list.***

We have added this in the References.

Furthermore, the authors discussed on the possibility that a repeated cycle of Pten-mediated Notch activation and Notch-mediated Pten repression underlies the asymmetric generation of RPC and neurons. ***This discussion is too speculative, because a major input to activate Notch is probably its ligand Delta, which is omitted in Fig. S6.***

We modified the figure by adding Delta/Jagged as a major activating signal for Notch, and have moved it to Figure 7 of revised manuscript.

12. In the discussion, ***the authors should discuss on how Akt activation inhibits Notch signaling****, in particular, the interaction between NICD and other components, CBF1 and MAML1, by citing related references.*

We discuss about the potential molecular mechanisms of Akt-induced NICD transcription complex regulation in Discussion (page 12) with related references.

13. In the introduction (or discussion), ***the authors should mention previous literatures*** *to report the roles of PTEN/PI3K-Akt in retina development, for example, Pimentel et al., (2002) Dev Biol. 247, 295-306; Kim et al. (2008) Gene and Dev 22, 3147-3157; Park et al. (2008) Science 322, 963-966.*

We mention about these papers in Results (page 5) and also added them in References.

2nd Editorial Decision 07 November 2011

Thank you for submitting your revised manuscript to the EMBO Journal. Your study has now been seen by the original referee #2 and the comments are provided below. As you can see, the referee appreciates the introduced changes and supports publication here. Before proceeding with acceptance of your paper for publication in the EMBO Journal, the referee raises a few minor issues that should be resolved.

Thank you for submitting your paper to the EMBO Journal

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Editor
The EMBO Journal

REFEREE REPORTS

Referee #2:

I have reviewed the revised manuscript submitted by Jo et al, which is entitled "Pten coordinates retinal neurogenesis by regulating Notch signaling". The authors have revised the manuscript in accordance with referees' comments and the revised manuscript is now greatly improved. However, I have a few question and request below.

(1) I am still confusing with the authors' explanation on new Fig. S3B. In the revised manuscript, the authors have changed its legend sentences. In the new legend, red, yellow and blue columns indicate the percentage of the number of TUNEL(+), TUNEL(+)/lacZ(+), and TUNEL(+)/lacZ(-) cells to the total number of retinal cells, respectively. If this is the case, these TUNEL percentages are higher in the Pten-cko retina than in the Pax6-aCre retina regardless of the presence of LacZ. The TUNEL percentage of Pten-deficient cells (LacZ(+)-derivative) seems to be equivalent to that of Pten-normal cells (LacZ(-)-derivative) in the Pten-cko retina. However, this situation is different from the description in the text. For example, in line 21 of page 6, the authors mentioned "There are indeed fewer apoptotic cells among the P5 Pten-deficient cells, which were LacZ-positive, than among the LacZ-negative normal retinal cells (Fig. S3)" and "The predominance of Pten-deficient retina might be a reflection of their enhanced survival or their non-autonomous induction of cell death in neighboring wild-type retinal cells". I guess that the description of the original manuscript is correct? Please confirm this point again.
(2) In Fig. S6B, hes1 mRNA expression level seems to be not different between in the Pten-cko retina than in Pax6-aCre retina. Please show a more valid image, if hes1 mRNA is decreased in the Pten-cko retina.

2nd Revision - authors' response 08 November 2011

Referee #2 :

I have reviewed the revised manuscript submitted by Jo et al, which is entitled "Pten coordinates retinal neurogenesis by regulating Notch signaling". The authors have revised the manuscript in accordance with referees' comments and the revised manuscript is now greatly improved. However, I have a few question and request below.

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As it is shown in the pictures (A) and graph (B), the average number of total TUNEL-positive cells in P5 *Pten-cko* retinas was higher than that of littermate *Pax6-aCre* retinas. The TUNEL-positive cells in the post-natal retinas account for the elimination of developmentally over-produced retinal neurons, which failed to form synaptic connections to their targets.

Larger numbers of retinal neurons were produced in *Pten-cko* mouse retinas than those of *Pax6-aCre* littermate retinas during development owing to the enhanced proliferation and neuronal differentiation in *Pten*-deficient RPCs (Figure 2A and 4; Figure S4 and S5). For example, more retinal ganglion cells (RGCs) accumulated in the GCL of P0 *Pten-cko* retina upon their birth (Figure S4A; DAPI-positive cells in GCL). However, their synaptic targets, such as superior colliculus (SC) and dorsal lateral geniculate nucleus (dLGN), are unaffected by Cre-mediated recombination, and the numbers of cells in *Pten-cko* SC and dLGN are indistinguishable from those of *Pax6-aCre* littermates (data not shown). Therefore, the final number of *Pten-cko* mouse RGCs that can survive by forming stable synaptic connections to their target is not significantly different from those of *Pax6-aCre* littermate mice (Figure 3A and 3B). This incompatibility of increased RGC number and

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unchanged SC and dLGN cell number in *Pten-cko* retinas may result in more RGCs fail to find their synaptic partners and die from apoptosis. Supporting this, there're more TUNEL-positive cells in RGCs in P1 *Pten-cko* retinas than littermate *Pax6-aCre* retinas (data not shown). The enhanced cell death of RGCs in *Pten-cko* retinas then subsequently results in death of more interneurons that also need to connect to RGC for their survival. Therefore, the elevated numbers of dying cells in *Pten-cko* retinas than Pax6-aCre reflect the post-natal adjustment of neuronal cell number by synaptic connection.

The TUNEL percentage of Pten-deficient cells (LacZ(+)-derivative) seems to be equivalent to that of Pten-normal cells (LacZ(-)-derivative) in the Pten-cko retina. However, this situation is different from the description in the text. For example, in line 21 of page 6, the authors mentioned "There are indeed fewer apoptotic cells among the P5 Pten-deficient cells, which were LacZ-positive, than among the LacZ-negative normal retinal cells (Fig. S3)" and "The predominance of Pten-deficient retina might be a reflection of their enhanced survival or their non-autonomous induction of cell death in neighboring wild-type retinal cells". I guess that the description of the original manuscript is correct? Please confirm this point again.

We have reformatted the graph, which has an X-axis that represents lacZ-positivity of TUNEL-positive cells. As explained above, the numbers of TUNEL-positive cells in P5 *Pten-cko* retinas were higher than those of *Pax6-aCre* littermate retinas by 2.4-folds (two left column). The TUNEL-positivity of lacZ-negative cells in *Pten-cko* retinas was significantly higher (15-folds) than that of *Pax6-aCre* littermate retinas (two middle columns). The TUNEL-positivity of lacZ-positive cells, which are potential *Pten*-deficient cells, in the *Pten-cko* retinas was moderately increased by 1.8-folds (two right columns). Therefore, the description as "The predominance predominance of *Pten*-deficient retinal cells in the post-natal *Pten-cko* retina might be a reflection of their enhanced survival or their non-autonomous induction of cell death in neighboring wild-type retinal cells" is correct according to our results that show specific and robust elevation of TUNEL(+);lacZ(-)/lacZ(-) wiltype cells in the *Pten-cko* retinas.

(2) In Fig. S6B, hes1 mRNA expression level seems to be not different between in the Pten-cko retina than in Pax6-aCre retina. Please show a more valid image, if hes1 mRNA is decreased in the Pten-cko retina.

We have exchanged with other images that show more clearly the difference of *Hes1* expression.

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