# Aquaporin regulates cell rounding through vacuole formation during endothelial-to-hematopoietic transition

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**Original submission**

First decision letter

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MS TITLE: Aquaporin regulates cell rounding through vacuole formation during endothelial-to-hematopoietic transition

AUTHORS: Yuki Sato, Mugiho Shigematsu, Maria Shibata-Kanno, Sho Maejima, Chie Tamura, and Hirotaka Sakamoto

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPressand click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee’s comments, and we will look over this and provide further guidance.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

*Advance summary and potential significance to field*

The article by Yuki Sato et al. describes, in the avian model, the role of aquaporins during delamination of first intraembryonic hematopoietic cells. Using in vivo and in vitro approaches, fine cellular and molecular techniques, the authors decipher the mechanisms that drive, in the aortic floor, the transition between flat endothelial cells and rounded hematopoietic cells that protrude in the vessel lumen. They postulate that the modification of the cellular morphogenesis is dependent on water flux through mobilization of aquaporin channels. These findings bring a pertinent novelty that will permit to more clearly decipher the emergence of hematopoietic cells in the embryo.

*Comments for the author*

The article by Yuki Sato et al. describes, in the avian model, the role of aquaporins during delamination of first intraembryonic hematopoietic cells. Using in vivo and in vitro approaches, fine cellular and molecular techniques, the authors decipher the mechanisms that drive, in the aortic floor, the transition between flat endothelial cells and rounded hematopoietic cells that protrude in the vessel lumen. They postulate that the modification of the cellular morphogenesis is dependent on the water flux through mobilization of aquaporin channels. These findings bring a pertinent novelty that will permit to more clearly decipher the emergence of hematopoietic cells in the embryo. Find below my comments.

L46-47: Françoise Dieterlen-Lièvre works have to be cited among the references. She was the first to discover the intraembryonic origin of hematopoietic cells in vertebrates (Dieterlen-Lièvre and Martin 1981 Dev Biol 88, 180-191; Godin et al. 1993 Nature 364, 67-70).

L76: Jaffredo et al. 2010 is missing in the reference list.

L89: "dorsal aortic floor": the oxymoron that associates dorsal and floor may be confused for the readers. It is true that the truncal part of the aorta is dorsal by comparison with the ventral aorta that emerges from the heart, but as the authors often refer to roof (or dorsal) and floor (or ventral) regions of the vessel, I would not write "dorsal aorta" but only "aorta". In this way, on L107, the authors wrote "dorsal aortic endothelial cells" while they only observed the aortic floor.

Fig 1: Concerning the percentage of Runx1+ cells in the aortic floor, I am surprised to read around 80% at E2.5 because the illustrating picture does not correlate with this number!

AQP1 is localized in the plasma and vacuole membranes:
L109-110: I do not understand the sentence "We identified this structural cavity as being distinct from the vacuole" as I do not understand the difference between "arrows" and "stars" in the Fig 2F. What are these structures and is it important to mention them?

AQP1 promotes ectopic vacuole formation and cell rounding:
It would be interesting to know whether roof cells that ectopically overexpress AQP1 detach and circulate at E4; I am agreed with the authors concerning the difficulty to optically access to the aorta at this stage, however, a time-lapse focused on vitelline vessels (as movie 3) would permit to record fluorescent circulating cells.

Did the authors perform AQP1 overexpression on the aortic floor? It would be interesting to know whether the electroporation induces an increase of vacuolated cells ventrally with or without an increase of Runx1+ hematopoietic cells.

Fig 3A: the sections illustrating the electroporation using AQP1(R196H)-mRFP show much more Runx1 cells than other above images: is it because the gene transfer efficacy was better than for the two other plasmids and could also explain why eGFP cells are also more numerous in the vicinity of the aorta?

Fig 4C and movie 2: How can we be sure that we really observe vacuole formation and not membrane protrusions?

L170: replace "a few days" by "3 days" as mentioned in methods.

AQP channels are redundantly required for HEC rounding:
I do not understand on Fig 6I why the percentage of mRFP+/Runx1+ cells at E5 is less important than at E4? The absence of AQPs abolishes the ability of hematopoietic cells to become round and detach from the aortic floor: thus, for me, it would be logical to get an accumulation of more or less flat hematopoietic cells ventrally in the aorta and an increased percentage at E5!

As the mRFP+/Runx1+ cells are not release in the circulation and stay among the aortic ventral endothelium, what do they become later in ontogeny? It would be interesting to check transverse sections at E6-7 to observe their behavior and maybe to focus on the development of paraaortic hematopoietic foci in this condition.

Fig S5A: I am not convinced by these ISH pictures, the staining is more or less at the level of the background!

Fig 6B: what are the mRFP+ cells beneath the aorta in the bottom picture and that are not present in the above image?

L208: I believe that "redundant" is not the appropriate word: I would prefer "concomitant".

Fig 3E and 6E: In these two pictures, the authors refer to vacuole sizes. In Fig 6E the measure unit is µm2. Is it the same unit in Fig3E? If it is the case, can the authors explain why the sizes of control vacuoles are so different between Fig 3E (±10-13) and Fig 6E (±4).

Discussion:
As AQPs do not seem to be involved in hematopoietic induction but more in hematopoietic cell delamination from the aortic floor, the authors have to discuss the involvement of AQPs in another processes of delamination as the epithelial-mesenchymal transition.

L276-278: The authors claim that the effects of multiple KO on hematopoietic stem cell colony formation cannot be experimentally shown in avian model. Nevertheless, in vitro experiments were performed a long time ago to demonstrate the ability of aortic hematopoietic clusters to form different hematopoietic lineages (Cormier et al. 1986 Dev Biol 118, 167-175; Cormier and Dieterlen-Lièvre 1988 Development 102, 279-285).

Material and methods:
L316: Precise that DRAQ5 stains nuclei.

In vitro EHT: Do the "slight modifications" mentioned by the authors concern the composition of the used medium? I think that its composition has to be added in the text.

References:
Add "Jaffredo et al. 2010" in the list.

Reviewer 2

*Advance summary and potential significance to field*

The paper by Sato et al is an interesting paper that aims at characterizing the molecular mechanisms underlying the lineage transition of hemogenic endothelial cells (HECs) into blood. The authors mainly focus on the changes in cell morphology during this endothelial-to-hematopoietic transition (EHT) and use the avian model with a combination of in vivo and in vitro assays. The main findings are the following: 1) Large vacuoles are formed transiently in HECs; 2) AQP1 is localized in the vacuoles; 3) Overexpression (OE) of AQP1 in cells that are non hemogenic induces a cell

morphology change reminiscent of the EHT; 4) Loss of AQP impairs the EHT. From these findings the authors conclude that the morphological remodeling of HECs occurring during EHT is regulated by the influx of H2O. The question addressed is undoubtedly important and timely, as little is known about the molecular regulation of the EHT and the manuscript focuses on an unexplored, and probably unexpected, angle, ensuring it is a novel and fascinating story. However, it lacks clarity in the use of terminology characterization of the cell populations targeted or observed, and the interpretation of the results. There are some claims that are made that are not supported by the data provided, and there are data that are not well explained or fully interpreted. Below are some points that are left unanswered and need to be addressed to grant a publication in Development.

*Comments for the author*

Major points

1) The term vacuole in and of itself is vague. The vacuoles are the central element of the paper, yet they are totally uncharacterized. The only characterization method the authors used to identify that these are indeed vacuoles is the lack of incorporation of constitutive GFP. In fact, the authors mention a report that untagged fluorescent proteins are excluded from vacuoles. Are they excluded from any other intracellular compartments?

The authors should provide a more thorough characterization of these vacuoles using markers of the principal organelles to prove or exclude that these are not endosomes, lysosomes, autophagosomes, etc. In particular, the authors mention the presence of other smaller vesicles present in the endothelium of the floor and roof that are eGFP negative and identify them as endosomes. While no characterisation (e.g. staining) is provided to prove this assertion, it is clear that it is quintessential to better define the vacuoles to support their claims, in particular the main message of the paper. The authors should also explore whether markers usually associated with vacuoles in other systems, such as VMN1, are expressed on the membrane lining the vacuoles.

The authors also try to distinguish vacuoles and cavities, mentioning that the formation of vacuoles that are distinct from cavities. First no characterization of the cavities (or vacuoles as mentioned) is provided. Second the relationship between cavities and vacuoles is also not demonstrated or clear and the differences are not described. For example, do cavities still form in the AQP knockdown model?

2) The authors claim that the cell rounding occurring during EHT and regulated by AQP is a RUNX1-independent process. The data provided however do not support this claim, as it is solely based on absence of RUNX1 staining. Any claim regarding the RUNX1-independence of this process requires thorough experiments in a RUNX1 null background, as this process could be simply downstream of RUNX1. As such the relationship between RUNX1 and AQPs should be better investigated.

3) The authors describe the use of non-HECs for their overexpression experiments. No proof is given that these are indeed non-hemogenic. On the contrary some of the data (in particular the video) look like there are rounded cells present in the non-HEC although it is unclear if these are dividing cells or cells undergoing the EHT. Better characterization of this population would bolster the authors findings. Related to this, the authors note that AQP1 expression is not exclusive to HECs and is expressed in endothelial cells throughout the dorsal aorta regardless of their position, hence also in non-HECs. What then mediates the HEC specific influx of H20? Why then does OE of AQP1 cause an EHT when physiological expression in non-EHT cells does not? Is it related to RUNX1? The authors should address this in the discussion.

4) Another area where terminology must be improved regards the output of the morphological changes. The authors should be careful with their claims about HSCs, as no characterization is provided to indicate that the cells indicated are HSCs or even blood cells. In order to claim that these are hematopoietic cells or that an EHT (and not just cell-rounding) is occurring, some form of characterization is required. A staining for a pan-hematopoietic marker (e.g. CD45 or other chick/quail equivalents) would be good. This would help to add quantification of changes in hematopoietic output, an essential indicator of the robustness/occurrence of the EHT across their

genetic manipulations. In fact quantification of the hematopoietic output using their in vitro system is important to evaluate changes in the EHT in response to their over expression and knock down perturbations.

5) The analysis of the RNA sequencing data that is included is incomplete and very vaguely described. More extensive explanation of these results would be useful. For example, does the sequencing data point ant any cytoskeletal rearrangements, as it would be expected? If so, can they validate them? For example, is the formation of this cavity the independently regulated by cytoskeletal changes? Can the authors block cytoskeletal polymerization and still see vacuole formation and EHT?

6) Throughout the paper, there is a lack of explanation/clarity the descriptions of the experimental methods that were selected and why in the results section. Two specific examples are listed below, but the entire results section should be closely considered for clear description of the methods employed and rationale behind their selection.

a) The authors mention the use of several models/reporters throughout the results section; however, it is unclear why these models (e.g. Lyn-mCherry and mRFP-CAAX) are used. A brief in context explanation would be beneficial for the reader.

b) The results from Figure 1D are mentioned in the text however no description of the methods used to obtain them are included. This would help the reader understand the results and also bolster their validity.

Other points
1) The authors mention that AQP1 is downregulated by E5, is this in all endothelial cells? Is this due to AQP1 positive cells undergoing the EHT and entering the bloodstream?
2) Does the AQP KD affect the timing of the EHT? Should the authors look earlier or later?
3) In the discussion the authors suggest that the process is dose-dependent based on their KD data requiring KD of multiple AQP genes. Can this conclusion be made based on this data? Or is this simply an indication of molecular redundancy?
4) The authors discuss that the localization of AQP1 suggests that “water accumulation begins in the basal space of HECs, which triggers the invagination of the basal membrane followed by internalization of the cavities into the vacuoles. Did the authors quantify the basal vs. apical localization of AQP1 on the membrane to support this assertion? A plot showing this data would be beneficial. Additionally, this is the first mention of the cavities being internalized into the vacuoles. Were the authors able to find evidence of this?
5) Nuclear staining would be beneficial for Figure 1.
6) Is there a statistically significant difference in the vacuole size for RUNX1+ and RUNX1- cells in the floor?
7) The authors mention that vacuoles were observed in the hemispherical and nearly spherical cells, but not in the fully rounded cells. A plot quantifying this would be helpful.
8) It would be beneficial if the authors could provide quantification of the vacuoles for the in vitro data.
9) Quantification of the proportion of AQP1-mRFP cells that become round is needed.

Reviewer 3

*Advance summary and potential significance to field*

The paper by Sato et al. reports the role of Aquaporins, a family of proteins localized to the plasma membrane that allow movement of water, glycerol and urea across cell membranes. More particularly, this paper addresses the role of Aquaporin1 (AQP1), in the formation of large vacuoles in endothelial cells to drive endothelial-to-haematopoietic transition (EHT).

The authors utilize ex vivo and in vivo approaches in the avian embryo.

First, they used GFP transgenic chick embryos to localize and measure vacuoles that are formed during EHT based on the fact that vacuoles are devoid of a GFP signal. They show that vacuoles in the aortic floor are bigger than those present in the roof and frequently associated with Runx1 positive cells, a transcription factor involved in EHT.

Second, using a custom anti AQP1 antibody, they showed that AQP1 was present in the cell and vacuole membranes both in light and electron microscopy.

Third, overexpression of WT or mutant AQP1 forms electroporated in the aortic roof shows that the AQP1 WT form induces large vacuoles in endothelial cells whereas the AQP1 mutant form fails to do it.

Fourth, ectopic expression of AQP1 in the vitelline vessels of the area pellucida, immediately lateral to the embryonic area, shows rounding and detachment of cells that reach the circulation and travel into the vessels.

Fifth, the authors used a previously published cell culture system recapitulating EHT and haematopoietic production to show that the system expresses AQP1 and forms vacuoles. They also performed transcriptomes of AQP1+ vs AQP1- endothelial cells and found an up-regulation of mechanical sensors and osmotic channels in the AQP1+ population.

Sixth: they performed CRISPR/Cas9 loss of function of AQP1 in the embryonic aorta and found no effect. They completed the approach by performing multiple Aquaporins CRISPR/Cas knock out e.g., 1, 5, 8, 9 and found a considerable reduction of the vacuoles in transfected cells.

This is an interesting piece of work that shed new light into the endothelial-to-haematopoietic transition. It suggest an uncoupling between the change of form and the haematopoietic program.

*Comments for the author*

This is an interesting piece of work that uses, precise, state of the art and cutting-edge approaches such as embryo electroporation to achieve gain and loss of function in ovo, live imaging to track the changes operating in cells and next generation sequencing to identify changes in transcriptome.

However, several key elements are missing before the work is suitable for publication in Development.

1° The anti AQP1 antibody is a custom one. It’s specificity to AQP1 vs the other AQP members should be shown using a classical approach such as Western blot. In the same line, the in situ data of AQP member in FigS5 are not very convincing. Better images are awaited.

2° An extensive expression analysis of AQP1 together with markers of haemogenic endothelium and haematopoietic cells such as Runx1 and CD45 from E2.5 to E5 is necessary to approach the complexity of the EHT and vacuole formation. In particular, the authors mention that the ratio of vacuolated Runx1+ cells decrease with time. This point is important to understand the role of AQP1 in EHT. Is the percentage of vacuolated vs non vacuolated cells evolve with time and are the haematopoietic cells produced from vacuolated endothelial cells different from those produced by non-vacuolated endothelial cells?

3° Albeit challenging, overexpression of AQP1 in the aortic roof is not very informative regarding the fate of the transfected cells because no functional haematopoietic marker has been used such as CD45. Consequently, it is difficult to associate the morphological changes driven by AQP1 overexpression with a putative change in gene expression and haematopoietic fate. Indeed, the authors mention that Runx1 expression is absent from the transfected cells but it may be that AQP1 could drive haematopoietic changes that does not need Runx1 expression.

More generally, the in vivo approaches the authors have undertaken are interesting and carry a technological added value but do not bring a lot of insights into the molecular programs driven by

AQP1. To gain insights into the role of AQP1 in driving EHT, this reviewer would suggest to use ex vivo culture approaches of endothelial cells. The best approach would be to overexpress AQP1 in non-haemogenic endothelial cells and perform either transcriptomics or quantitative PCR to reveal genes involved in EHT and haematopoietic commitment. Alternatively, the pre-somitic mesoderm culture system the authors have used (Yvernogeau et al., 2016) could be edited for AQP knock out, transfected cells FACS sorted and compared to non-edited cells for the transcriptomic expression. It will give a more global view of the changes operated by AQP expression. Since the authors have performed transcriptome analysis of the cells in vitro, it should be helpful to know which AQP they have found in the analysis.

4° the authors show round, circulating cells following overexpression of AQP1 in the vitelline vessels. It is of interest to know if the rounding is limited to arterial endothelial cells or can also be seen in veins and what is the identity of the circulating cells. Based on the number of round, circulating cells seen on the movie, it should be possible to FACS sort these cells and perform quantitative PCR to approach their identity.

5° Can the authors explain in more details the calculations they use to represent the % of Runx1+ cells in Figure 1. This reviewer does not understand whether this represents the % of Runx1+ cells among all ECs? Or is it the % of Runx1+ cells in the floor (or roof) of the aorta among the total Runx1+ cells…Please clarify this point.

In general, the authors often overstate or misinterpret the results regarding the current litterature:

Line 70: “In contrast, within amniote embryos, HECs begin rounding toward the apical side, and HSCs are released directly into the blood stream (Boisset et al., 2010)”

The quoted paper is using an ex vivo culture system therefore not showing a direct release into the blood stream.

Line 72-73 based on the fact that haematopoietic bud in two opposite directions in amniotes vs zebrafish, the authors state that the mechanisms should be different. It has been shown by many publications that it is not the case and due to the size of the aorta in the zebrafish (25 micrometers) it is impossible for the haematopoietic cells to bud into the lumen without plugging the vessel. This is the reason why the evolution has selected a budding outside the aorta. However haematopoietic cells produced in the aorta do form large vacuoles/vesicles that should have a similar role than those investigated here (Lancino et al., 2018).

Line 95: “Commonly observed small vesicles in both the floor and roof were endosomes yielded by the intrinsic endocytic activity of endothelial cells (Simionescu et al., 2002).”

This is an assumption for which the authors should provide definitive proof of this statement or town-down the sentence.

Line 229 “Runx1 is genetically required for vacuole formation”. It is an overstatement that contradicts previous author assertion according to which vacuole formation is Runx1 independent.

The authors are using AQP1 tools but, due to redundancies in protein functions, it is not clear which AQP(s) is playing a role. This point should be further investigated or at least discussed.

The authors mention that the AQP-dependant vacuole formation is required for haematopoietic cells to be released in the blood flow. Since para aortic foci are known to originate from aorta-derived haematopoietic cells that ingress in the aortic floor, do these para-aortic foci cells follow the same AQPs/vacuoles-dependant mechanism? Can the authors comment on that.

Minor points

Line 64 ◊ missing dot before “Given that HECs…”

Figure 1H: the legend states that quantification was done for stage E5, which is not included on the graph. Please, correct.

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**First revision**

Author response to reviewers' comments

We thank the reviewers and the editor for their insightful and thorough evaluation. We have addressed the reviewers' concerns and agree that their suggestions have resulted in a much improved paper. The reviewers wanted us to revise the manuscript to clarify the writing and to further elaborate and justify our conclusions. We have taken their advice and revised the paper extensively to make it clearer and more concise. The reviewers' comments are in italics below, followed by our response in normal type.

**Point-by-point response to the reviewers:**

Reviewer 1

*L46-47: Françoise Dieterlen-Lièvre works have to be cited among the references. She was the first to discover the intraembryonic origin of hematopoietic cells in vertebrates (Dieterlen-Lièvre and Martin 1981 Dev Biol 88, 180-191; Godin et al. 1993 Nature 364, 67-70).*

> Thanks for suggesting important references; Dieterlen-Lièvre and Martin (1981) and Garcia-Porrero et al (1995) have been added to line 49 in the revised manuscript.

*L76: Jaffredo et al. 2010 is missing in the reference list.*

> We have added Jaffredo et al. (2010) to the reference list, which was missing from the previous version as pointed out by the reviewer.

*L89: "dorsal aortic floor": the oxymoron that associates dorsal and floor may be confused for the readers. It is true that the truncal part of the aorta is dorsal by comparison with the ventral aorta that emerges from the heart, but as the authors often refer to roof (or dorsal) and floor (or ventral) regions of the vessel, I would not write "dorsal aorta" but only "aorta". In this way, on L107, the authors wrote "dorsal aortic endothelial cells" while they only observed the aortic floor.*

> In line 45, we have added the short explanation, "dorsal aorta (hereafter referred to as the aorta)" and in the following text, "dorsal aorta" in the previous version has been replaced by "aorta" in the revised manuscript.

*Fig 1: Concerning the percentage of Runx1+ cells in the aortic floor, I am surprised to read around 80% at E2.5 because the illustrating picture does not correlate with this number!*

> Particularly at E2.5, Runx1 signal intensities varied between cells. To help identification of Runx1-positive cells with low fluorescence signal intensity, DRAQ overlay images are added to Fig. 1A and I, and Runx1-positive cells are marked with arrows.

*AQP1 is localized in the plasma and vacuole membranes:*
*L109-110: I do not understand the sentence "We identified this structural cavity as being distinct from the vacuole" as I do not understand the difference between "arrows" and "stars" in the Fig 2F. What are these structures and is it important to mention them?*

> As the reviewer pointed out, the explanation of the cavity was not clear in the previous version, so we have extensively revised Figs 1 and 2, by adding new data for a detailed explanation (Figs 1J–M, 2F–I, 6G and H, lines 101-107, 270-272) and Discussion (lines 344-356).

*AQP1 promotes ectopic vacuole formation and cell rounding:*

*It would be interesting to know whether roof cells that ectopically overexpress AQP1 detach and circulate at E4; I am agreed with the authors concerning the difficulty to optically access to the aorta at this stage, however, a time-lapse focused on vitelline vessels (as movie 3) would permit to record fluorescent circulating cells.*

Movies of the vitelline vessels in control, AQP1-, and AQP1(R196H)-overexpressed embryos are newly added to the supplemental data (Movie 1) and described in lines 167-170.

*Did the authors perform AQP1 overexpression on the aortic floor? It would be interesting to know whether the electroporation induces an increase of vacuolated cells ventrally with or without an increase of Runx1+ hematopoietic cells.*

The vacuole size of AQP1-overexpressing cells in the aortic floor did not differ from that of cells located in the aortic roof.

*Fig 3A: the sections illustrating the electroporation using AQP1(R196H)-mRFP show much more Runx1 cells than other above images: is it because the gene transfer efficacy was better than for the two other plasmids and could also explain why eGFP cells are also more numerous in the vicinity of the aorta?*

Distribution of Runx1-expressing cells varies with longitudinal position and embryos. We have confirmed that the Runx1-positive cells shown in Fig. 3A do not express mRFP tag. This suggests that the increase in Runx1-positive cells seen in the Fig. 3A lower panels, is not an effect of AQP1 (R196H) overexpression. A description of the electroporated cells around the aorta was added to the legend of revised Fig. 3 (lines 976-978).

*Fig 4C and movie 2: How can we be sure that we really observe vacuole formation and not membrane protrusions?*

The localization pattern of F-actin cytoskeleton in control and AQP1-overexpressing cells was added to the revised figure, showing that F-actin-rich processes are missing in AQP1-overexpressing cells (Fig. 4D and E, lines 197-203).

*L170: replace "a few days" by "3 days" as mentioned in methods.*

As suggested by the reviewer, the pointed words have been revised.

*AQP channels are redundantly required for HEC rounding:*
*I do not understand on Fig 6I why the percentage of mRFP+/Runx1+ cells at E5 is less important than at E4? The absence of AQPs abolishes the ability of hematopoietic cells to become round and detach from the aortic floor: thus, for me, it would be logical to get an accumulation of more or less flat hematopoietic cells ventrally in the aorta and an increased percentage at E5!*

The cells counted in E4 are the percentage of Runx1+/RFP+ cells in the total number of vascular endothelial cells, but these Runx1+/RFP+ cells are not necessarily AQPs multiple knockout cells because CRISPR/Cas9 genome editing is not 100% efficient. We believe that the AQP-intact/Runx1+/RFP+ cells seen at E4 have normally detached from the aortic floor by E5. In the Results section, we add sentences to explain the likely reason for the Runx1+/RFP+ decrease at E5 (lines 286-291).

*As the mRFP+/Runx1+ cells are not release in the circulation and stay among the aortic ventral endothelium, what do they become later in ontogeny? It would be interesting to check transverse sections at E6-7 to observe their behavior and maybe to focus on the development of paraaortic hematopoietic foci in this condition.*

Although this is a very important point, it was difficult to analyze the knockout cells in later stage embryos due to the decreased expression of fluorescent reporters. We have tracked the electroporated cells at later stages using the Tol2 transposon vector (Sato et al., Dev. Biol., 305, p616-624, 2007, doi:10.1016/j.ydbio.2007.01.043.), but currently we have not established a

transposon vector system that can be used to detect Cas9-transfected cells due to enormously large vector size. The developmental fate of multiple AQP1-deficient/Runx1+ cells at later stages remains to be determined. For these reasons, we were unable to analyze the knockout cells at E6-7, but we have confirmed that these AQP1-deficient/Runx1+ cells have contributed to the para-aortic foci at E5 (revised Fig. 6H, lines 278-280).

*Fig S5A: I am not convinced by these ISH pictures, the staining is more or less at the level of the background!*

We have replaced the AQP9 *in situ* hybridization results with a better image. Our staining is consistent with the chicken embryo ISH database, GEISHA ID: AQP5.UApcr, http://geisha.arizona.edu/geisha/photo.jsp?url_path=/geisha/photos/&id=47421; GEISHA ID: AQP8.UApcr, http://geisha.arizona.edu/geisha/photo.jsp?url_path=/geisha/photos/&id=47410; GEISHA ID:AQP9.UApcr, http://geisha.arizona.edu/geisha/photo.jsp?url_path=/geisha/photos/&id=47405. The original description was misleading and has been changed to state that AQP5, 8. 9 is expressed in both surrounding mesenchyme and endothelial cells in the aorta (Fig. S6 legend, lines 94-95 in Supplementary information).

*Fig 6B: what are the mRFP+ cells beneath the aorta in the bottom picture and that are not present in the above image?*

Electroporation into the aortic endothelium is performed on the precursors of the lateral plate mesoderm in primitive streak at HH4, so the transfected cells will be found in the mesenchyme underneath the aorta. A description of the electroporated cells underneath the aorta was added to the legend of Fig. 6 (lines 1055-1056).

*L208: I believe that "redundant" is not the appropriate word: I would prefer "concomitant".*

The pointed word has been revised as suggested by the reviewer.

*Fig 3E and 6E: In these two pictures, the authors refer to vacuole sizes. In Fig 6E the measure unit is µm2. Is it the same unit in Fig3E? If it is the case, can the authors explain why the sizes of control vacuoles are so different between Fig 3E (±10-13) and Fig 6E (±4).*

As the reviewer pointed out, an important description of the increased vacuole size by AQP1 overexpression was omitted, so we have added descriptions of the effect of excess AQP1 on vacuole size in the Results (lines 144-147).

*Discussion:*
*As AQPs do not seem to be involved in hematopoietic induction but more in hematopoietic cell delamination from the aortic floor, the authors have to discuss the involvement of AQPs in another processes of delamination as the epithelial-mesenchymal transition.*

Descriptions of the involvement of AQP in cell delamination events, including EMT, are added to the Introduction in the revised manuscript (lines 65-67). We have also revised the descriptions of the RNA-seq analysis with respect to EMT (lines 243-246).

*L276-278: The authors claim that the effects of multiple KO on hematopoietic stem cell colony formation cannot be experimentally shown in avian model. Nevertheless, in vitro experiments were performed a long time ago to demonstrate the ability of aortic hematopoietic clusters to form different hematopoietic lineages (Cormier et al. 1986 Dev Biol 118, 167-175; Cormier and Dieterlen-Lièvre 1988 Development 102, 279-285).*

Thanks for the important point, we have added to the discussion the prospective use of the avian model for *in vitro* analysis of multilineage potential (lines 388-391).

*Material and methods:*
*L316: Precise that DRAQ5 stains nuclei.*

In the Materials and Methods section, DRAQ5 was described as a stain for nuclei (lines 434-435).

*In vitro EHT: Do the "slight modifications" mentioned by the authors concern the composition of the used medium? I think that its composition has to be added in the text.*

The only slight change is the use of Dispase. The original paper (Yvernogeau et al., Development 143, p1302-1312, 2016) uses a different tissue separation method with a supplemental movie (https://movie.biologists.com/video/10.1242/dev.126714/video-1), which can be seen by comparing the two.

*References:*
*Add "Jaffredo et al. 2010" in the list.*

Thank you for pointing this out. We have added it.

Reviewer 2
*Major points*

*1) The term vacuole in and of itself is vague. The vacuoles are the central element of the paper, yet they are totally uncharacterized. The only characterization method the authors used to identify that these are indeed vacuoles is the lack of incorporation of constitutive GFP. In fact, the authors mention a report that untagged fluorescent proteins are excluded from vacuoles. Are they excluded from any other intracellular compartments? The authors should provide a more thorough characterization of these vacuoles, using markers of the principal organelles to prove or exclude that these are not endosomes, lysosomes, autophagosomes, etc. In particular, the authors mention the presence of other smaller vesicles present in the endothelium of the floor and roof that are eGFP negative and identify them as endosomes. While no characterisation (e.g. staining) is provided to prove this assertion, it is clear that it is quintessential to better define the vacuoles to support their claims, in particular the main message of the paper. The authors should also explore whether markers usually associated with vacuoles in other systems, such as VMN1, are expressed on the membrane lining the vacuoles.*

We performed immunostaining for Lamp1 and LC3 in GFP tg embryos (Fig. S1) and found that our description in the previous manuscript was incorrect. In the revised version, we have removed the incorrect description and replaced with confirmed fact (lines 109-112). Since in vertebrate cells, VMN1 is known to localize to the endoplasmic reticulum and autophagosomes (Rapolo et al., JBC, 282, P37124-37133, 2007), we have not tested VMN1 localization.

*The authors also try to distinguish vacuoles and cavities, mentioning that the formation of vacuoles that are distinct from cavities. First no characterization of the cavities (or vacuoles as mentioned) is provided. Second the relationship between cavities and vacuoles is also not demonstrated or clear and the differences are not described. For example, do cavities still form in the AQP knockdown model?*

The cavity data originally shown in the previous Fig. 2 have been moved to Fig. 1, and new data representing a 3D model of the cavity have been added in the revised version (Fig. 1K and L, lines 104-107 and 939-943). Additional data showed that cavity-like structures were not formed in multiple AQP-knockout cells (Fig. 6H, lines 270-271 and 1063-1066). And the relationship between cavity and vacuoles has been discussed (lines 344-357).

*2) The authors claim that the cell rounding occurring during EHT and regulated by AQP is a RUNX1-independent process. The data provided however do not support this claim, as it is solely based on absence of RUNX1 staining. Any claim regarding the RUNX1-independence of this process requires thorough experiments in a RUNX1 null background, as this process could be simply downstream of RUNX1. As such the relationship between RUNX1 and AQPs should be better investigated.*

AQP1 is expressed more broadly than Runx1 in aortic endothelial cells (Fig. 2), overexpression of AQP1 leads to vacuole formation and cell rounding without upregulation of Runx1 (Fig. 3), and multiple AQP knockouts induce vacuole formation and cell rounding even in cells expressing Runx1 (Fig. 6). Based on these original results, we concluded that vacuole formation and cell rounding by AQP occur in a Runx1-independent manner in the previous manuscript. In the revised manuscript, a new loss-of-function experiment using CRISPR/Cas9 genome editing of Runx1 have been added (Fig. S7). This result did not change our initial conclusion, suggesting a possible different role of Runx1 for morphological EHT in avian embryos compared to mouse (lines 272-275 in Results, lines 299-300; 307-314 in Discussion).

*3) The authors describe the use of non-HECs for their overexpression experiments. No proof is given that these are indeed non-hemogenic. On the contrary some of the data (in particular the video) look like there are rounded cells present in the non-HEC although it is unclear if these are dividing cells or cells undergoing the EHT. Better characterization of this population would bolster the authors findings. Related to this, the authors note that AQP1 expression is not exclusive to HECs and is expressed in endothelial cells throughout the dorsal aorta regardless of their position, hence also in non-HECs. What then mediates the HEC specific influx of H20? Why then does OE of AQP1 cause an EHT when physiological expression in non-EHT cells does not? Is it related to RUNX1? The authors should address this in the discussion.*

In the revised manuscript, AQP1-expressing cells in the aortic floor have been described in more detail using the hematopoietic cell marker, CD45 (Fig. 2F–I, lines 122-131). And a detailed description of the expression pattern of AQP1 has been added (Fig. S3, lines 119-122). For the area of time-lapse observation, a reference (Bollerot et al., 2005, doi.org/10.1016/j.modgep.2005.05.003) showing distribution of non-hemogenic (Runx1-) vitelline vessels has been added to the revised manuscript (line 177). We have also added time-lapse observations of mitotic cell rounding in control embryos (Movie. 4) and described that vacuoles do not form during transient cell rounding (lines 185-188). It has been known that homotetramer formation is essential for the water-gating function of AQPs. We believe that overexpression of AQP1 increased the absolute number of homotetramers and caused the increase in water permeability. This is described in the revised Discussion (lines 319-323)。

*4) Another area where terminology must be improved regards the output of the morphological changes. The authors should be careful with their claims about HSCs, as no characterization is provided to indicate that the cells indicated are HSCs or even blood cells. In order to claim that these are hematopoietic cells or that an EHT (and not just cell-rounding) is occurring, some form of characterization is required. A staining for a pan-hematopoietic marker (e.g. CD45 or other chick/quail equivalents) would be good. This would help to add quantification of changes in hematopoietic output, an essential indicator of the robustness/occurrence of the EHT across their genetic manipulations. In fact, quantification of the hematopoietic output using their in vitro system is important to evaluate changes in the EHT in response to their over expression and knock down perturbations.*

In the revised manuscript, we have added data showing that Runx1 is expressed earlier than CD45 and is maintained in the CD45-expressing cells in the aortic clusters (Fig. 2G-I). Therefore, similar to mouse embryos, Runx1 is available as a marker for both HECs and hematopoietic cells in avian embryo models (lines 122-126). Currently, we cannot technically distinguish AQP1-overexpressing cells from endogenously AQP1-upregulated cell populations in vitro. To accurately characterize AQP1 overexpressing and multiple AQP knockout cells, we need to optimize the selection system (drug or FACS). Practically, it is not possible to perform both loss-of-function and gain-of-function experiments and subsequent characterization (e.g. RNA-seq) within a few months for revision. Instead, in the revised manuscript, we have added CD45 staining of AQP1-overexpressed embryos (Fig. 4F, G, J-L, lines 204-214, discussed in lines 371-378) and a discussion of the possibility of assessing hematopoietic potentials using conventional cell lineage analysis methods (lines 388-391).

*5) The analysis of the RNA sequencing data that is included is incomplete and very vaguely described. More extensive explanation of these results would be useful. For example, does the*

*sequencing data point ant any cytoskeletal rearrangements, as it would be expected? If so, can they validate them? For example, is the formation of this cavity the independently regulated by cytoskeletal changes? Can the authors block cytoskeletal polymerization and still see vacuole formation and EHT?*

The description of the RNA-seq analysis has been extensively revised to provide more details (Fig. 5L and M, lines 233-237). Although, insights into cytoskeletal regulation and EMT were not obtained (lines 243-246), we have added data on the F-actin cytoskeleton in AQP1-overexpressing cells (Fig. 4D and E) and described the results in the revised manuscript (lines 197-203).

*6) Throughout the paper, there is a lack of explanation/clarity the descriptions of the experimental methods that were selected and why in the results section. Two specific examples are listed below, but the entire results section should be closely considered for clear description of the methods employed and rationale behind their selection.*
*a) The authors mention the use of several models/reporters throughout the results section; however, it is unclear why these models (e.g. Lyn-mCherry and mRFP-CAAX) are used. A brief in context explanation would be beneficial for the reader.*
*b) The results from Figure 1D are mentioned in the text however no description of the methods used to obtain them are included. This would help the reader understand the results and also bolster their validity.*

The revised version has been modified to improve clarity (lines 101-102 and 140-141, regarding a; lines 925-927, regarding b).

*Other points*

*1) The authors mention that AQP1 is downregulated by E5, is this in all endothelial cells? Is this due to AQP1 positive cells undergoing the EHT and entering the bloodstream?*

AQP1 is endothelial cell-specific because it co-localizes with QH1 (Figs S2C and S3). At E5, AQP1 expression is absent throughout the aorta (Fig. S3B). This cannot be explained simply by the release of AQP1-positive cells into the circulation. The revised version includes this discussion (lines 340-342).

*2) Does the AQP KD affect the timing of the EHT? Should the authors look earlier or later?*

We observed embryos at day 2.5, but it is difficult to see the morphological difference between control and multiple AQP knockout cells because Runx1-expressing cells are not rounded at this stage (Fig. 1A). Regarding later stages, due to the difficulty in detecting mRFP-positive genome-edited cells, analysis of embryos later than day 5 is not performed.

*3) In the discussion the authors suggest that the process is dose-dependent based on their KD data requiring KD of multiple AQP genes. Can this conclusion be made based on this data? Or is this simply an indication of molecular redundancy?*

A more detailed discussion of AQP dose dependence is included in the revised version (lines 319-323).

*4) The authors discuss that the localization of AQP1 suggests that "water accumulation begins in the basal space of HECs, which triggers the invagination of the basal membrane followed by internalization of the cavities into the vacuoles. Did the authors quantify the basal vs. apical localization of AQP1 on the membrane to support this assertion? A plot showing this data would be beneficial. Additionally, this is the first mention of the cavities being internalized into the vacuoles. Were the authors able to find evidence of this?*

The mechanism of accumulation of water molecules at the basal side of the plasma membrane should be investigated in light of the localization of AQP5, 8, and 9 in normal embryos. However, this cannot be verified at this time because anti-AQP5, 8, and 9 antibodies are not

available for avian embryos. We have supplemented our discussion with an example of the mechanism of vacuole formation in Schlemm's canal facilitated by basal water accumulation (lines 344-356).

*5) Nuclear staining would be beneficial for Figure 1.*

DRAQ5 staining image was added to the revised Fig. 1A–D (lower panels).

*6) Is there a statistically significant difference in the vacuole size for RUNX1+ and RUNX1- cells in the floor?*

There was no statistical difference between the two.

*7) The authors mention that vacuoles were observed in the hemispherical and nearly spherical cells, but not in the fully rounded cells. A plot quantifying this would be helpful.*

Quantitative data were added to revised Fig. 1M (lines 105-107 and 942-943).

*8) It would be beneficial if the authors could provide quantification of the vacuoles for the in vitro data.*

Quantification of sporadically eGFP-labeled cells has been added to the revised Fig. 5F–I (lines 223-227 and 1034-1038).

*9) Quantification of the proportion of AQP1-mRFP cells that become round is needed.*

Quantitative data has been added to revised Fig. 3E (lines 153-155 and 986-987).

*Reviewer 3*

*1° The anti AQP1 antibody is a custom one. It's specificity to AQP1 vs the other AQP members should be shown using a classical approach such as Western blot. In the same line, the in situ data of AQP member in FigS5 are not very convincing. Better images are awaited.*

Western blotting showing the specificity of the AQP1 antibody has been added to Fig. S2. With respect to in situ hybridization, AQP5, 8, and 9 are also expressed in the mesenchyme surrounding the dorsal aorta (short description has been added to revised Fig. S6 legend, lines 94-95 in Supplementary information). Our results are consistent with the in situ hybridization database of chick embryos, GEISHA ID: AQP5.UApcr, http://geisha.arizona.edu/geisha/photo.jsp?url_path=/geisha/photos/&id=47421; GEISHA ID: AQP8.UApcr, http://geisha.arizona.edu/geisha/photo.jsp?url_path=/geisha/photos/&id=47410; GEISHAID:AQP9.UApcr, http://geisha.arizona.edu/geisha/photo.jsp?url_path=/geisha/photos/&id=47405.

*2° An extensive expression analysis of AQP1 together with markers of haemogenic endothelium and haematopoietic cells such as Runx1 and CD45 from E2.5 to E5 is necessary to approach the complexity of the EHT and vacuole formation. In particular, the authors mention that the ratio of vacuolated Runx1+ cells decrease with time. This point is important to understand the role of AQP1 in EHT. Is the percentage of vacuolated vs non vacuolated cells evolve with time and are the haematopoietic cells produced from vacuolated endothelial cells different from those produced by non-vacuolated endothelial cells?*

Immunostaining data for CD45/Runx1 and CD45/AQP1 have been added to provide more detail on changes in EHT over time (Fig. 2G-I, lines 122-131). Whether hematopoietic cells derived from vacuolated endothelial cells differ from those derived from non-vacuolated endothelial cells is an important question raised by the reviewer, but it is technically difficult to specifically label vacuole-free cells for isolation. As a remaining issue, we have added a short sentence to the Discussion (lines 362-364).

*3° Albeit challenging, overexpression of AQP1 in the aortic roof is not very informative regarding the fate of the transfected cells because no functional haematopoietic marker has been used such as CD45. Consequently, it is difficult to associate the morphological changes driven by AQP1 overexpression with a putative change in gene expression and haematopoietic fate. Indeed, the authors mention that Runx1 expression is absent from the transfected cells but it may be that AQP1 could drive haematopoietic changes that does not need Runx1 expression.*
*More generally, the in vivo approaches the authors have undertaken are interesting and carry a technological added value but do not bring a lot of insights into the molecular programs driven by AQP1. To gain insights into the role of AQP1 in driving EHT, this reviewer would suggest to use ex vivo culture approaches of endothelial cells. The best approach would be to overexpress AQP1 in non-haemogenic endothelial cells and perform either transcriptomics or quantitative PCR to reveal genes involved in EHT and haematopoietic commitment. Alternatively, the pre- somitic mesoderm culture system the authors have used (Yvernogeau et al., 2016) could be edited for AQP knock out, transfected cells FACS sorted and compared to non-edited cells for the transcriptomic expression. It will give a more global view of the changes operated by AQP expression. Since the authors have performed transcriptome analysis of the cells in vitro, it should be helpful to know which AQP they have found in the analysis.*

CD45 immunostaining was performed on AQP1-overexpressing cells and the results have been added to the revised Fig. 4G, K and L (lines 204-208 and 1015-1023). As the reviewer points out, the molecular cascade induced by AQP1 overexpression needs to be clarified to understand cellular responses to the water influx. We are currently investigating genes whose expression is altered in AQP1-overexpressing cells and performing further loss-of-function and gain-of-function experiments on these molecules, which will be reported in a separate paper.

*4° the authors show round, circulating cells following overexpression of AQP1 in the vitelline vessels. It is of interest to know if the rounding is limited to arterial endothelial cells or can also be seen in veins and what is the identity of the circulating cells. Based on the number of round, circulating cells seen on the movie, it should be possible to FACS sort these cells and perform quantitative PCR to approach their identity.*

We have added new results in the revised Fig. 3H showing that ectopic cell rounding also occurs in the cardinal vein as a result of AQP1 overexpression (lines 163-167). We attempted to collect circulating AQP1-overexpressing cells from day 3 embryos and subjected them to FACS, but were unable to obtain sufficient numbers of transfected cells for qRT-PCR analysis. Instead, CD45 immunostaining was performed on circulating AQP1-expressing cells and the results are shown in the revised Fig. 4K and L (lines 204-214, discussed in lines 371-378).

*5° Can the authors explain in more details the calculations they use to represent the % of Runx1+ cells in Figure 1. This reviewer does not understand whether this represents the % of Runx1+ cells among all ECs? Or is it the % of Runx1+ cells in the floor (or roof) of the aorta among the total Runx1+ cells…Please clarify this point.*

The labeling of the Y-axis in Fig. 1F and the description of Fig 1E and F in the figure legend (lines 925-927) have been revised for clarity.

*In general, the authors often overstate or misinterpret the results regarding the current litterature: Line 70: "In contrast, within amniote embryos, HECs begin rounding toward the apical side, and HSCs are released directly into the blood stream (Boisset et al., 2010)"*
*The quoted paper is using an ex vivo culture system therefore not showing a direct release into the blood stream.*

In the revised manuscript, we have replaced "bloodstream" in the original manuscript with "aortic lumen" (line 77).

*Line 72-73 based on the fact that haematopoietic bud in two opposite directions in amniotes vs zebrafish, the authors state that the mechanisms should be different. It has been shown by many publications that it is not the case and due to the size of the aorta in the zebrafish (25 micrometers)*

*it is impossible for the haematopoietic cells to bud into the lumen without plugging the vessel. This is the reason why the evolution has selected a budding outside the aorta. However haematopoietic cells produced in the aorta do form large vacuoles/vesicles that should have a similar role than those investigated here (Lancino et al., 2018).*

We have changed the original text to read the cellular mechanism has not been elucidated in avian embryos (lines 77-78).

*Line 95: "Commonly observed small vesicles in both the floor and roof were endosomes yielded by the intrinsic endocytic activity of endothelial cells (Simionescu et al., 2002)."*
*This is an assumption for which the authors should provide definitive proof of this statement or town-down the sentence.*

Since the immunostaining results for the lysosomal marker, Lamp1, and the autophagosome marker, LC3B, suggested that the vacuoles were not degradative vesicles, this was noted in the revised manuscript (Fig. S1, lines 109-112) and the original text was deleted.

*Line 229 "Runx1 is genetically required for vacuole formation". It is an overstatement that contradicts previous author assertion according to which vacuole formation is Runx1 independent.*

This phrase has been replaced by a more euphemistic writing style in the revised manuscript (lines 302-304).

*The authors are using AQP1 tools but, due to redundancies in protein functions, it is not clear which AQP(s) is playing a role. This point should be further investigated or at least discussed.*

We have revised the Discussion to describe the limitation of our study in lines 327-330 and 382-385.

*The authors mention that the AQP-dependant vacuole formation is required for haematopoietic cells to be released in the blood flow. Since para aortic foci are known to originate from aorta-derived haematopoietic cells that ingress in the aortic floor, do these para-aortic foci cells follow the same AQPs/vacuoles-dependant mechanism? Can the authors comment on that.*

We have added new data to show that cells in the para-aortic foci do not express AQP1 (Fig. 2H, lines 130-131). We have also replaced the original Fig. 6H with another image to show that multiple AQP knockout has no effect on migration to the para-aortic foci (lines 279-280).

*Minor points*

*Line 64 ◊ missing dot before "Given that HECs…"*
*Figure 1H: the legend states that quantification was done for stage E5, which is not included on the graph. Please, correct.*

Thank you for pointing this out. We have corrected them.

**Corrections other than those pointed by the reviewer:**

Alignments of CRISPR/Cas9 genome editing mutations have been revised in Fig. S5A and S6B because the previous version did not adequately represent deletions due to the default setting of the Genetyx software, which aligns sequences to minimize gaps.

Second decision letter

MS ID#: DEVELOP/2022/201275

MS TITLE: Aquaporin regulates cell rounding through vacuole formation during endothelial-to-hematopoietic transition

AUTHORS: Yuki Sato, Mugiho Shigematsu, Maria Shibata-Kanno, Sho Maejima, Chie Tamura, and Hirotaka Sakamoto

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is very positive and we would like to publish a revised manuscript in Development, provided that the referees' remaining minor comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the refereeâ€™s comments, and we will look over this and provide further guidance.

Reviewer 1

*Advance summary and potential significance to field*

The article by Yuki Sato et al. describes, in the avian model, the role of aquaporins during delamination of first intraembryonic hematopoietic cells. Using in vivo and in vitro approaches, fine cellular and molecular techniques, the authors decipher the mechanisms that drive, in the aortic floor, the transition between flat endothelial cells and rounded hematopoietic cells that protrude in the vessel lumen. They postulate that the modification of the cellular morphogenesis is dependent on water flux through mobilization of aquaporin channels. These findings bring a pertinent novelty that will permit to more clearly decipher the emergence of hematopoietic cells in the embryo.

*Comments for the author*

The revised manuscript DEVELOP/2022/201 275 by Sato et al. took account the most part of my queries and seriously modified the text (while, sometimes the corrected lines, mentioned in the response letter, did not correspond in the revised version). However, I always have a problem with in situ hybridizations on supplemental figure 6: the signal still remains faint! Looking at GEISHA database the authors mentioned to compare, on E5.5 chick sections (stage 26HH), only the AQP5 messenger is clearly expressed in the aortic floor but not AQP8 and 9. AQP5 and 9 are observed in the cardinal vein, AQP5 mRNAs are present in dorsal aortic branches, but AQP8 ISH does not show any significant staining in the aorta and the surrounding mesenchyme! Maybe that the difference can be explained by the different ages studied between the database and the Sato et al.’s study (E3.5), nevertheless, my opinion is that ISH will not convince the readers and has to be removed or substantially improved.

Reviewer 2

*Advance summary and potential significance to field*

The authors answered rather thoroughly to the points raised by this reviewer.

*Comments for the author*

The only point that still need to be addressed is their reference to HSC throughout the manuscript. While HSCs are generated via EHT, not all EHT events generate HSCs. As the authors have not tested the stemness of the EHT output they should refer to the cells as hematopoietic cells and not HSCs also in their abstract, introduction and discussion.

Reviewer 3

*Advance summary and potential significance to field*

This is the revised version of the manuscript by Sato et al.
The authors have done a very complete and documented job They have answered point by point to my questions and complete the manuscript by either performing new experiments (Western blot, immunohistochemistry on the section, new electroporation...) or changing the text and I thank them very much for that.
I regret that my suggestion to use the culture system to analyze in depth the biological question that the authors address with difficulties in the embryo was not followed, but still.
As it is, the work appears to be complete and very well conducted, although there are still some unanswered questions that the authors will have to answer in a future paper.
Basically, overexpression of aquaporin in the non-hemogenic venous endothelium, i.e., cardinal veins, triggers rounding but does not activate the hematopoietic program. In one case, the authors found an aquaporin+ CD45+ circulating cell, which I consider a fortunate coincidence. So, these data show that the rounding program and the hematopoietic program are uncoupled. We also learn that aquaporin is expressed both dorsally and ventrally in the aortic endothelium, but only the ventral endothelium undergoes endothelial to hematopoietic conversion meaning that the hemogenic endothelium will allow combining both vacuole production under the control of aquaporin and initiation of the hematopoietic program likely triggered by Runx1. How the two uncoupled programmes become associated to give rise to hematopoietic cells will certainly be a key question in the next future.
Based on the elements mentions above and the answers to my questions, I am happy to accept the manuscript.

*Comments for the author*

None

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**Second revision**

Author response to reviewers' comments

MS ID#: DEVELOP/2022/201275
MS TITLE: Aquaporin regulates cell rounding through vacuole formation during endothelial-to-hematopoietic transition

AUTHORS: Yuki Sato, Mugiho Shigematsu, Maria Shibata-Kanno, Sho Maejima, Chie Tamura, and Hirotaka Sakamoto

Decision: Revision - Minor; Decision Date: 3 April, 2023
Handiling editor: Dr. Hanna Mikkola
Corresponding Author: Yuki Sato

Authors response to reviewers:
We thank the reviewers for concerning our manuscript for publication in Development. We have revised the manuscript accordingly to the reviewers' suggestion. The reviewers' comments are in italics below, followed by our response in normal type.

Point-by-point response to the reviewers:
Reviewer 1
*The revised manuscript DEVELOP/2022/201 275 by Sato et al. took account the most part of my queries and seriously modified the text (while, sometimes the corrected lines, mentioned in the response letter, did not correspond in the revised version). However, I always have a problem with in situ hybridizations on supplemental figure 6: the signal still remains faint! Looking at GEISHA database the authors mentioned to compare, on E5.5 chick sections (stage 26HH), only the AQP5 messenger is clearly expressed in the aortic floor but not AQP8 and 9. AQP5 and 9 are observed in the cardinal vein, AQP5 mRNAs are present in dorsal aortic branches, but AQP8 ISH does not show any significant staining in the aorta and the surrounding mesenchyme! Maybe that the difference can be explained by the different ages studied between the database and the Sato et al.'s study (E3.5), nevertheless, my opinion is that ISH will not convince the readers and has to be removed or substantially improved.*

> Thank you for pointing this out. We checked the image in Fig. S6A on another monitor and found that the AQP8 signal was unclear, as the reviewer noted. We have replaced it with a better image in the revised version.

Reviewer 2
*The only point that still need to be addressed is their reference to HSC throughout the manuscript. While HSCs are generated via EHT, not all EHT events generate HSCs. As the authors have not tested the stemness of the EHT output, they should refer to the cells as hematopoietic cells and not HSCs also in their abstract, introduction and discussion.*

> We have revised the text to state hematopoietic cells instead of HSCs (lines 30 and 39 in the Abstract, lines 51, 53, 74, 77, and 84 in the Introduction, line 232 in the Results, lines 362 and 370 in the Discussion, and Fig. 6L).

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Third decision letter

MS ID#: DEVELOP/2022/201275

MS TITLE: Aquaporin regulates cell rounding through vacuole formation during endothelial-to-hematopoietic transition

AUTHORS: Yuki Sato, Mugiho Shigematsu, Maria Shibata-Kanno, Sho Maejima, Chie Tamura, and Hirotaka Sakamoto
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.