Cardiac function modulates endocardial cell dynamics to shape the cardiac outflow tract

Pragya Sidhwani, Dena M. Leerberg, Giulia L. M. Boezio, Teresa L. Capasso, Hongbo Yang, Neil C. Chi, Beth L. Roman, Didier Y. R. Stainier and Deborah Yelon
DOI: 10.1242/dev.185900

Editor: Benoit Bruneau

Review timeline
Original submission: 27 October 2019
Editorial decision: 14 November 2019
First revision received: 21 April 2020
Accepted: 27 April 2020

Original submission

First decision letter

MS ID#: DEVELOP/2019/185900

MS TITLE: Cardiac function modulates endocardial cell dynamics to shape the cardiac outflow tract

AUTHORS: Pragya Sidhwani, Giulia Boezio, Hongbo Yang, Neil Chi, Beth Roman, Didier Stainier, and Deborah Yelon

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.

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 to 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.

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

In the study, “Cardiac function modulates endocardial cell dynamics to shape the cardiac outflow tract”, Sidhwani et al. explore the mechanisms that regulate OFT formation with a focus on the endocardial compartment. Specifically, they claim that OFT endothelial cells derived from second

heart field progenitors proliferate in situ between 36 and 51 hours to enlarge the developing structure. In addition, they confirm previous observations (Rochon et al., 2018) that endothelium from aortic arch 1 (AA1) becomes incorporated into the OFT endocardium. Using mutants and morphants that affect contractility (tnnt2a and myh7) and blood flow (myh6), the authors demonstrate that pump function is essential for both OFT endocardial proliferation and AA1-derived endothelial incorporation, while flow supports AA1-mediated endothelial incorporation alone. Finally, the authors confirm a previous observation (Rochon et al., 2018) that the flow-responsive TGFb receptor Acvrl1/Alk1 is necessary for AA1-mediated endothelial incorporation into the OFT but dispensable for OFT endocardial proliferation.

Understanding how the OFT is established during development is of significant value to the community. The imaging is of high quality, the genetic tools used are appropriate, and the text is easy to follow and understand. However, the model for OFT endocardial development remains unclear and needs further refinement.

Comments for the author

1) In general terms, it is unclear when progenitor cells from PA2 stop giving rise to OFT endocardium and when OFT endocardial cells expand by proliferation.

Based on EdU and BrdU pulse chase, the claim is that OFT endocardial cells proliferate in situ to increase cell numbers from ~10 cells at 36 to ~20 at 51 hpf. However, this experiment cannot discern whether differentiated endocardial cells proliferate once in the OFT as suggested or if progenitor cells that give rise to the endothelium are proliferating prior to differentiation.

A previous report demonstrated that when nkx2.5+ progenitor cells located in pharyngeal arch (PA) 2 are labeled at 28 hpf, the signal can be visualized in all three OFT lineages, including endocardium, at 72 hpf (Paffett-Lugassy et al., 2017). However, when these cells stop being added to the OFT was not analyzed. Therefore, it is impossible to know if the EdU/BrdU signal at 51 hpf is from proliferative progenitors that subsequently turn on the endothelial reporter or if differentiated endothelial cells are dividing. Defining the time when nkx2.5+ progenitor cells stop being added to the OFT endocardium would help refine the model.

It appears that ~20 endocardial cells comprise the OFT at 51 hpf. What fraction of these derive from PA2 progenitor differentiation, endocardial cell proliferation, and AA1? This level of resolution would be very valuable for investigators attempting to analyze mutants with OFT endocardial deficiencies.

2) Can the authors clarify how they conceptualize the addition of AA1 endothelium to the OFT? Do they think it is added much like the SHF accretes new muscle to the poles of the heart tube? Alternatively, does the addition of more myocardial cells simply change the boundary of AA1 bifurcation such that the most proximal AA1 endothelial cells become incorporated into the OFT as the myocardium wraps around it? The term “endothelial displacement” was used in the title of Supp Movie 7 – is this different from active accretion?

Do the authors think that displacement of these AA1 endothelial cells is essential for normal OFT development? Acvr1 mutants have decreased endocardial cell numbers which they attribute to decreased AA1 endothelial cells displacement. Do Acvr1 mutants have normal myocardial cell numbers in their OFT? If so then this outcome would suggest that it is not merely a growing OFT myocardium that shifts the boundary of AA1 bifurcation, but that AA1 endothelial displacement is an active process that is required for OFT development.

In the last paragraph of the Results section, it is suggested that Acvrl1 functions downstream of cardiac function to regulate AA1 endothelial displacement. Can AA1 endothelial displacement be rescued in contractile mutants/morphants (tnnt2a or myh7) by overexpressing acvrl1 in the endothelium using the Tg(fli1a:acvrl1-MYC)pt516 strain?

Novelty:
1) The idea that endothelium comprising AA1 becomes incorporated into OFT endocardium was previously reported in Rochon et al., 2018. If the data presented here extend their findings, then it

would be helpful to include which experiments confirm the Rochon et al. data and what is new from this study.

2) Figure 8 (here) appears to be have been reported in Figure 4 of Rochon et al., 2018. If the data here extend the findings of Rochon et al. regarding the Alk1/Acvrl1 requirement for supporting AA1 endothelial addition to the OFT then it will be helpful to state.

Minor:

The inclusion of middle section counts was unclear as the authors report total cell counts for all of their data.

Reviewer 2

Advance summary and potential significance to field

In their manuscript, Sidhwani et al. present a thorough and carefully carried out analysis on the morphogenesis of the cardiac outflow tract (OFT). They use zebrafish genetics and high-resolution confocal imaging to examine cell behaviors involved in OFT morphogenesis. The authors show that the growth of OFT relies on a cumulative outcome of changes in cell number cell shape and cell size of both myocardial and endocardial cells. The authors then focus their analysis on evaluating the mechanisms that regulate the OFT endocardial expansion. They find that loss of ventricular contractility and blood flow (tnnt2 and myh7) impairs both endocardial proliferation and addition whereas atrial contractility (myh6) and the TGFβ receptor Acvrl1 are required for recruiting endocardial cells from neighboring vessels to the OFT but have no effects on endocardial cell proliferation. The authors conclude that endocardial proliferation and addition are influenced by distinct modes of cardiac function-dependent regulation during OFT endocardial expansion. Overall, the manuscript is clearly written. The data are of high-quality and provide new insights into how cardiac function influences various cell behaviors during OFT morphogenesis.

Comments for the author

Specific Comments:
1. The authors show that OFT endocardial expansion relies on the proliferation of local endocardial cells as well as the incorporation of cells from neighboring vessels. It would be helpful if the authors could comment on the relative contributions of these cell behaviors to OFT endocardial expansion.
2. Using the photoconvertible transgenic zebrafish, the authors show that AA1 is an external source of endothelial cells that contribute to the growth of OFT endothelium. Could the authors comment on (1) whether AA1 endothelial cells also contribute to other vessels and (2) whether ventricular endothelium contribute to OFT endothelium, if so their distribution in the OFT.
3. The authors made an interesting observation that both proliferation and addition are impaired in tnnt2 and myh7 mutants whereas myh6 deficiency affects only the recruitment of endothelial cells from AA1. Because myh6 encodes an atrial-specific form of myofibril protein and the mutant ventricle does contract, the authors conclude that altered flow parameters affects cell addition. It would greatly strengthen the data if the author could provide quantitative measurement of ventricular contractility in myh6 mutants during the stages relevant for OFT growth. It would also be helpful if the authors could examine OFT endocardial cell behaviors in a different model where flow and ventricular contractility are uncoupled (by genetic or pharmacological manipulation).
4. If indeed endocardial proliferation and recruitment are regulated by distinct modes of cardiac function-dependent regulation, it would be helpful to know whether they are also regulated at different critical time windows.
Other suggestions:
1. Figure 2K-M, the red fluorescence in the OFT (arrow) do not look convincing. It would be nice if the authors could provide a better image.
2. The authors used a single asterisk to denote statistical significance. It would be less confusing if the authors could follow the convention symbols (* $p<0.05$, ** $p<0.01$ etc.)

Reviewer 3

Advance summary and potential significance to field

In this manuscript by Sidhwani et al the authors analysed outflow tract (OFT) development using the zebrafish model. The authors describe that while the OFT myocardium is expanding between 36 and 51 hpf by accretion of SHF cells, a similar expansion occurs in the endocardium. Endocardial cell number in the OFT increases over this time period. Using EdU labeling for cell proliferation and photoconversion for cell migration, they find that both cell proliferation and cell migration contributes to the expansion of the OFT endocardium. Using mutants that lack contractility (tnnt2, myh7) or have reduced blood flow (myh6), the authors observed that endocardial cell migration depends on blood flow while endocardial proliferation depends on contractility. By using genetic mutants in combination with morpholino knock downs, the authors conclude that while Klf2 is not required for endocardial expansion, acvrl1 is required for endocardial cell migration into the OFT.

This manuscript is very well written and the figures are of very high quality supporting the authors' main conclusions. The novelty in the work relates to the finding that while both cardiac contraction and blood flow regulate endocardial growth, they both seem to do so by regulating different processes (namely proliferation versus migration/accretion respectively). While there is some redundancy with previous published work which is acknowledges by the authors, this work looks at all the various aspects (cell migration, proliferation flow dynamics, contractile force) in relation to OFT development. This is highly relevant for a better understanding of congenital heart defects and more specifically to those related to defects in OFT development, which are the most severe congenital heart defects.

Comments for the author

Major concern:
1. My only major concern relates to the interpretation of the Acvrl1 mutant/knock-down experiments. The authors conclude from the observation that endocardial cell numbers are reduced when Acvrl1 is not present that endothelial cell accretion to the OFT is impaired and they suggest a cell-autonomous role for Acvrl1 in endothelial cells. Do the authors have additional observations (e.g. p-Smad2,3 staining) that Tgf-beta signalling is restricted to endothelial cells (versus myocardial cells)? It would be important to know whether myocardial cell accretion to the OFT is affected when Acvrl1 is absent, since this might indirectly result in reduced endocardial cell numbers.

Minor concerns:

1. At the end of the first paragraph of the results section the authors write: 'Importantly, our data indicate that the OFT widens during this interval, and that this increase in width corresponds with both myocardial and endocardial cellular accumulation along the circumference of the OFT.'. Although the widening of the OFT at 51 hpf compared to 36 hpf is clearly visible in the images, it is nowhere mentioned in the text nor indicated in the figure. It would be helpful for the reader to introduce this observation more clearly.

2. Most graphs in the figures are bar charts and do not show the spreading of the data points. It would be more informative to overlap the data points with the bar charts whenever possible.

First revision

Author response to reviewers' comments

We are grateful to all three reviewers for their positive feedback regarding our work. We are especially thankful for their view that our manuscript presents "*a thorough and carefully carried out analysis on the morphogenesis of the cardiac outflow tract*" and "*is highly relevant for a better*

understanding of congenital heart defects." We also appreciate the reviewers' thoughtful suggestions for strategies to strengthen our manuscript's value. We have now modified our manuscript in accordance with their input, both by adding new data and by amending the text. Notably, our revised submission includes five new figures (Supplementary Figures S2, S11, S13, S14, and S15), and updated sections of the text are highlighted throughout the attached document. We feel that these changes have substantially enhanced the significance and clarity of our manuscript, and we thank the reviewers for their assistance with this improvement. Our point-by-point responses to the reviewers' comments are assembled below.

(Finally, please note the addition of Dr. Dena M. Leerberg and Ms. Teresa L. Capasso as authors on the manuscript, as they both contributed to the work involved in its revision.)

Response to Reviewer #1:

We are grateful for Reviewer #1's positive assertion that "*understanding how the OFT is established during development is of significant value to the community*," and we are pleased by Reviewer #1's assessment that our "*imaging is of high quality, the genetic tools used are appropriate and the text is easy to follow and understand*." Reviewer #1 also offered valuable suggestions for approaches to refine our model for the mechanisms underlying OFT endocardial development. Specifically:

1. Reviewer #1 wonders whether the EdU+/BrdU+ cells that we observed in the OFT endocardium represent the division of proliferative progenitor cells or differentiated endocardial cells. Since our EdU/BrdU assays evaluated proliferation occurring between 36 and 51 hpf, we chose to address this issue by evaluating how many "late-differentiating" endothelial cells are added to the OFT endocardium during this timeframe: in the context of this experiment, "late" is defined as differentiation after 36 hpf. Specifically, we photoconverted the entirety of the endothelium in *Tg(kdrl:dendra)* embryos at 36 hpf and then examined whether the OFT endocardium contained any cells exhibiting green, but not red, fluorescence at 51 hpf. In 10 out of 11 embryos analyzed, we detected "green-only" cells in the OFT endocardium; however, in these cases, only a small number (~3-5) of "green-only" cells were evident (Fig. S2). Interestingly, these late-differentiating cells were always found clustered near each other (n=10/10) and were typically located within the distal half of the OFT (n=9/10). In contrast, the observed EdU+/BrdU+ cells (Figs 2A-C,E-G; S7A,B) appear to be distributed in both the proximal and distal portions of the OFT and were not necessarily clustered together. Taking into account all of these data, we suspect that some of our observed EdU+/BrdU+ cells could represent the division of proliferative progenitor cells, but that it is also likely that some of our observed EdU+/BrdU+ represent the division of some differentiated endocardial cells. In our revised manuscript, these data are included in a new Supplementary Figure (Fig. S2) and are discussed in our revised results and discussion sections (pp. 9, 10, 17, 18).

In addition, as requested by Reviewer #1, our revised discussion section includes commentary on the relative contributions of late-differentiating cells, endocardial proliferation, and endothelial displacement to the expansion of the OFT endocardium between 36 and 51 hpf (pp. 17-18). Although it is clear that all three of these sources contribute to the observed increase in OFT endocardial cell number, it is difficult to estimate their relative proportions of involvement without understanding how they overlap: late-differentiating cells or cells from AA1 may be among the proliferating cells, and late-differentiating cells may also enter the OFT via AA1. In future studies, beyond the scope of this revision, we plan to perform high-resolution timelapse imaging to track the behavior of individual endothelial cells and resolve the relationships between late-differentiating cells, proliferating cells, and cells from AA1 between 36 and 51 hpf. We mention this future direction in our revised discussion section (pp. 17-18).

2. Reviewer #1 asks whether endothelial addition to the OFT is an active process "*much like the SHF accretes new muscle to the poles of the heart tube*" or a passive one where "*addition of more myocardial cells simply change the boundary of AA1 bifurcation*". We appreciate the importance of distinguishing between these possibilities. Based on prior timelapse analyses demonstrating that endothelial cells migrate from the cranial vasculature into the heart (Rochon et al., 2016), we favor the former, active model, and we make reference to this previously documented cell migration throughout our text (pp. 3, 9, 12, 14, 15). However, our current experiments do not directly document active cell migration: this would require tracking cell behavior via live timelapse imaging, beyond the scope of this revision. In our manuscript, we therefore carefully refrain from using terms

that imply active cell movement, and we refer to the behavior of AA1 cells joining the OFT as "addition", "incorporation", "movement", or "displacement", instead of "migration". Our revised discussion section makes reference to the importance of pursuing timelapse analyses in order to resolve this issue in the future (pp. 17-18). We acknowledge that we cannot rule out the latter, passive model. However, it is interesting to note that *tnnt2a* mutants seem to exhibit a normal number of OFT myocardial cells (Fig. S5E) and a normal myocardial volume (Fig. 3V) while also displaying a substantial defect in the addition of AA1 endothelial cells (Fig. 6T), suggesting that that myocardial accretion is not coupled to endothelial displacement. We make reference to this point in our revised text (p. 47).

Reviewer #1 also asks whether "*displacement of AA1 endothelial cells is essential for normal OFT development*". We would suggest that it is indeed important, not only for insuring an appropriate number of OFT endocardial cells but also for achieving normal endocardial morphology, as suggested by the aberrant cell numbers and morphologies of the OFT endocardium in the *myh6* and *acvrl1* loss-of-function scenarios (Figs 4, 7), both of which exhibit defective endothelial displacement (Figs 6, 8). Our revised manuscript reinforces this point further by including a new figure (Fig. S11) demonstrating a decrease in OFT endocardial volume in *acvrl1* morphants (p. 14). However, Reviewer #1 also brings up an important question to consider: how does *acvrl1* function affect OFT myocardial cell number? In our revised manuscript, we include a new figure (Fig. S13) demonstrating that *acvrl1* morphants exhibit a reduced number of OFT myocardial cells (p. 15). This observation raises interest in how *acvrl1* influences the accretion of OFT myocardium and whether the OFT endocardial defects in *acvrl1*-deficient embryos might be a secondary consequence of their myocardial phenotype. To investigate these issues, we chose to test whether expression of wild-type *acvrl1* in the endothelium could rescue the endocardial and/or myocardial phenotypes in the *acvrl1* mutant OFT. Strikingly, we find that expression of the transgene *Tg(fli1:acvrl1-MYC)* (Laux et al., 2013) can rescue both endocardial and myocardial cell number in the OFT of *acvrl1* mutants (Fig. S14). As described in our revised manuscript (p. 15), these data indicate that *acvrl1* functions within the endothelium to regulate OFT endocardial growth. Additionally, these data suggest that the OFT myocardial defects in *acvrl1* mutants are either secondary to their endothelial displacement defects or due to another role of *acvrl1* in the endothelium.

Additionally, Reviewer #1 asks if AA1 endothelial displacement can be rescued by expression of *Tg(fli1:acvrl1-MYC)* in the absence of cardiac function. As suggested by Reviewer #1, we examined the impact of *Tg(fli1:acvrl1-MYC)* in *tnnt2a* morphants, and we found that expression of *Tg(fli1:acvrl1-MYC)* did not rescue their OFT endocardial defects (Fig. S15). As described in our revised results section (pp. 15-16), we conclude that restoration of *acvrl1* function in the endothelium is insufficient to recapitulate the influence of cardiac function on OFT expansion, and we presume that additional function-dependent factors act in concert with *acvrl1* expression to promote OFT growth. One possibility is that blood flow may regulate both the expression of *acvrl1* and the dissemination of the Acvrl1 ligand Bmp10, which is thought to be transported via blood flow from the heart to the arterial vasculature (Laux et al., 2013). This idea is described in our revised discussion section (p. 19).

3. Reviewer #1 asks for clarification of the degree of novelty of our data regarding addition of AA1 cells to the OFT endocardium. As noted in our original text, prior work (Rochon et al., 2016) had demonstrated that cells from the arterial vasculature can migrate into the heart between 24 and 48 hpf. Our data (Figs 2J,Q-V, S1) extend these prior findings by evaluating whether cells from AA1 come to occupy the OFT during the specific timeframe of our analyses, between 36 and 51 hpf. We clarify this point in our revised results section (p. 9).

4. Reviewer #1 also asks for clarification of the degree of novelty of our data regarding the role of *acvrl1* in promoting addition of AA1 cells to the OFT. Specifically, Reviewer #1 asks how our data in Figure 8 are distinct from the data shown in Figure 4 of the prior study by Rochon et al. (2016). As noted in our original text, prior work (Rochon et al., 2016) had found that *acvrl1* mutants demonstrate reduced endothelial migration toward the heart between 24 and 48 hpf. Our data (Fig. 8) extend these prior findings by evaluating the role of *acvrl1* in promoting addition of AA1 cells to the OFT during the specific timeframe of our analyses, between 36 and 51 hpf. We clarify this point in our revised results section (p. 15). In addition, our work provides several novel insights regarding the role of *acvrl1* during OFT morphogenesis. Notably, we uncover previously unappreciated roles of *acvrl1* in promoting the expansion of the OFT endocardium and the accretion of the OFT myocardium, and we

demonstrate that *acvrl1* acts within the endothelium for both of these functions (Figs 7, 8, S10, S11, S13, S14; pp. 14-16).

5. Reviewer #1 raises a question about the value of including middle section cell counts in our figures, since we also report total cell counts. We appreciate the reviewer's point that total cell counts are more inclusive; at the same time, we feel that providing the number of cells in a typical section conveys additional information about how the circumference of the tissue is changing over time (as in Fig. 1C,D,G,H,K,L,O,P,S,T,W,X). In our revised results section, we have added text to clarify the value of including this type of data (pp. 7-8).

Response to Reviewer #2:

We are grateful for Reviewer #2's appreciation that our "*data are of high-quality and provide new insights into how cardiac function influences various cellular behaviors during OFT morphogenesis*." Reviewer #2 also pointed out several ways in which we could strengthen the clarity and significance of our manuscript. Specifically:

1. Reviewer #2 requests that we comment on the relative contributions of endocardial proliferation and incorporation of endothelial cells to the expansion of the OFT endocardium. As mentioned above (Reviewer #1, point #1), we agree that it is worthwhile to discuss this point further, and we have added commentary on the relative contributions of late-differentiating cells, endocardial proliferation, and endothelial displacement to our revised discussion section (pp. 17-18). As stated above (Reviewer #1, point #1), while we can demonstrate that late-differentiating cells, proliferating cells, and cells from AA1 all participate in OFT endocardial expansion, it is difficult to estimate their relative proportions of involvement without understanding how they overlap with each other. With this in mind, our revised discussion also notes that future studies will be necessary to resolve the relationships of these three sources of OFT endocardial cells (pp. 17-18).

2. Reviewer #2 asks whether AA1 endothelial cells contribute to vascular tissues other than the OFT endocardium. Although we did not rigorously score contributions to other locations, careful analysis in a prior study (Rochon et al., 2016) demonstrated that AA1 endothelial cells migrated into both the ventricle and the OFT between 24 and 48 hpf, and we cite this work in our manuscript (p. 9).

Reviewer #2 also asks whether cells from the ventricular endocardium contribute to the OFT endocardium. To evaluate this, we attempted to label the ventricular endocardium using photoconversion in *Tg(kdrl:dendra)* embryos at 36 hpf. However, in all of these instances, we inadvertently labeled a portion of the OFT endocardium as well. Thus, it seems that the orientation of the embryo at 36 hpf that allows us to visualize both the OFT and the ventricle is not ideal for specifically labeling only the ventricle. We apologize for these technical constraints; in the future, we plan to combine live imaging and multicolor labeling approaches in order to assess whether ventricular endocardial cells move into the OFT endocardium.

3. Reviewer #2 requests that our revised manuscript include a quantitative assessment of ventricular contractility in *myh6* mutants. Our original text stated that the *myh6* mutant ventricle contracts at a normal rate and cited our prior publication (Berdougo et al., 2003) in support of this point. In our prior publication (Berdougo et al., 2003), we reported ventricular heart rates of 150 ± 26 bpm in *myh6* mutants (n=5) and 139 ± 22 bpm in their wild-type siblings (n=10) at 48 hpf. We appreciate that it would be valuable for the reader to have this type of quantitative definition of a "normal rate" in our results section. Therefore, instead of restating our prior data (Berdougo et al., 2003), our revised text includes a new data set from heart rate measurements performed in our current *myh6* strain at 51 hpf. As reported in our revised results, we found ventricular heart rates of 120 ± 10 bpm in *myh6* mutants (n=8) and 126 ± 9 bpm in their wild-type siblings (n=7) at 51 hpf (p. 12).

In addition, we agree with Reviewer #2 that it would be helpful for us to assess the OFT endocardium in other scenarios in which flow and contractility are uncoupled. Thus far, our attention in this regard has focused on analysis of *gata1* morphants, which were previously reported to exhibit reduced shear forces (Vermot et al., 2009), and *gata2* morphants, which were previously reported to exhibit both reduced shear forces and reduced retrograde flow (Vermot et al., 2009). As reported in our

original manuscript (Fig. 4I-L), the number of OFT endocardial cells appears normal in *gata1* morphants. In contrast, we did find a reduced number of OFT endocardial cells in *gata2* morphants (data not shown). However, this result is challenging to interpret, particularly because we and others (Dietrich et al., 2014) have noted that *gata2* morphants also exhibit a reduced ventricular heart rate by 51 hpf. Therefore, it is difficult to consider the *gata2* morphant phenotype as an example in which flow and contractility are uncoupled. Because of this interpretative uncertainty, we prefer to refrain from including the *gata2* morphant data in our revised manuscript. In future studies, beyond the scope of this revision, we plan to pursue alternative strategies for uncoupling flow and contractility, including surgical methods such as focal occlusion of the dorsal aorta (Serluca et al., 2002). We discuss the importance of this future direction in the discussion section of our revised manuscript (p. 18).

4. Reviewer #2 asks whether endocardial proliferation and endothelial recruitment occur during different intervals within the 36-51 hpf time window. We agree that it would be interesting to refine our understanding of when each of these processes occurs. In an effort to define the time window within which OFT endocardial cells proliferate, we performed immunostaining with an antibody against phosphorylated histone H3 (phH3) at a series of stages between 36 and 51 hpf. However, at each stage examined, we were unable to identify more than one phH3+ endocardial cell per embryo. We suspect that the cells marked by our EdU and BrdU analyses do not all divide simultaneously; in addition, as mentioned above (Reviewer #1, point #1), some of these cells may not be located in the endocardium at the time that they are dividing. In either case, we conclude that we are currently unable to refine the proliferation time window via analysis of fixed embryos. In future studies, beyond the scope of this revision, we plan to perform high-resolution timelapse analysis, as mentioned above (Reviewer #1, point #1), in order to attain a clearer understanding of the precise timing of the relevant proliferation and recruitment events, and we mention this in our revised discussion section (pp. 17-18).

5. Reviewer #2 points out that it is challenging to see the red fluorescence in the OFT in Figure 2K-M, and we acknowledge this difficulty. Unfortunately, labeling the OFT endocardium in these experiments is technically challenging: the constrained optical accessibility of the OFT at 36 hpf limits the resolution of our images of the photoconverted cells at this stage. We apologize for our inability to provide more satisfying images.

6. As suggested by Reviewer #2, we have adjusted the symbols used to indicate statistical significance in our figures. Throughout our revised manuscript, we have incorporated the following symbols: * to indicate $p<0.05$, ** to indicate $p<0.01$, *** to indicate $p<0.001$, and **** to indicate $p<0.0001$. This set of symbols is explained in our revised Materials and Methods section (p. 27).

Response to Reviewer #3:

We are grateful for Reviewer #3's positive evaluation that our "*manuscript is very well written and the figures are of very high quality supporting the authors' main conclusions.*" In addition, Reviewer #3 noted some aspects of our manuscript that would benefit from clarification or elaboration. Specifically:

1. Reviewer #3 wonders whether *acvrl1* is likely to play a cell-autonomous role in the endothelium during the process of OFT expansion. Along these lines, Reviewer #3 asks whether pSmad is found within the endothelium during relevant stages. Acvrl1 function is known to regulate pSmad1/5/8 activity, and pSmad1/5/8 has previously been detected in the arterial vasculature during our timeframe of interest (Laux et al., 2013). However, pSmad1/5/8 localization can also represent the activity of BMP receptor heterodimers containing the type I receptor Alk2 (rather than Acvrl1). Furthermore, detection of pSmad1/5/8 in the endothelium does not rule out potential activity of Acvrl1 in other locations. Therefore, to address the concerns raised by Reviewer #3, we pursued a series of experiments in order to evaluate the impact of *acvrl1* on the OFT myocardium and the location of *acvrl1* function during OFT expansion. In brief, and as described above (Reviewer #1, point #2), we demonstrated that *acvrl1* morphants exhibit a reduced number of OFT myocardial cells (Fig. S13, p. 15), and we demonstrated that expression of the transgene *Tg(fli1:acvrl1-MYC)* can rescue both endocardial and myocardial cell number in the OFT of *acvrl1* mutants (Fig. S14, p. 15). Thus, our data indicate that *acvrl1* functions within the endothelium to regulate OFT endocardial growth. Additionally, our data suggest that the OFT myocardial defects in *acvrl1* mutants are either secondary

to their endothelial displacement defects or due to another role of *acvrl1* in the endothelium.

2. As pointed out by Reviewer #3, the first section of our original results section did not explicitly describe changes in the width of the wild-type OFT between 36 and 51 hpf. We apologize for this oversight. Our revised results section now includes this point (pp. 7-8).

3. As requested by Reviewer #3, we have adjusted our bar graphs to include overlapping data points. This style of presentation has been incorporated into Figures 1, 2, 4, 7, S5, S9, S10, S12, S13, S14 and S15. Please note that we did not adjust the stacked bar graphs in Figures 5M, 7F, and S7I, since this style of graph is not amenable to display of overlapping data points. Additionally, we chose not to adjust the graphs reporting our volumetric analysis (Figs 3U-W, S4E, S11C); since these data points were acquired by constructing surfaces representative of the endocardial and myocardial walls, they represent estimates and not precise measurements.

Second decision letter

MS ID#: DEVELOP/2019/185900

MS TITLE: Cardiac function modulates endocardial cell dynamics to shape the cardiac outflow tract

AUTHORS: Pragya Sidhwani, Dena M. Leerberg, Giulia Boezio, Teresa L. Capasso, Hongbo Yang, Neil Chi, Beth Roman, Didier Stainier, and Deborah Yelon
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.

Reviewer 1

Advance summary and potential significance to field

The revision presented is clear and thoughtful, providing the community with new knowledge of outflow tract endocardial morphogenesis.

Comments for the author

No further revisions are needed.

Reviewer 2

Advance summary and potential significance to field

The authors have addressed all my concerns. I support the publication of the manuscript as it is.

Comments for the author

please see comments above.