osr1 couples intermediate mesoderm cell fate with temporal dynamics of vessel progenitor cell differentiation

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MS TITLE: osr1 couples intermediate mesoderm cell fate with temporal dynamics of vessel progenitor cell differentiation

AUTHORS: Elliot Perens, Jessyka Diaz, Agathe Quesnel, Amjad Askary, Gage Crump, and Deborah Yelon

Many apologies for the delay in obtaining the referees' reports on the above manuscript. 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 reviews are mixed with one reviewer considering that there are insufficient novel insights into how osr1 functions to consider publication in Development and two other reviews that are more enthusiastic. The more negative referee does make various suggestions about how he/she thinks the study can be improved as does one of the more positive referees. If you are able to revise the manuscript along the lines suggested, I will be happy receive a revised version of the manuscript. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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

Here, the authors continue their very nice previously published studies on the roles of hand2 in mesoderm development by examining the genetic intersect with osr1. The authors report phenotypes of osr1 mutant zebrafish, showing for the first time that osr1 is requisite for the emergence of pax2a+ intermediate mesoderm (IM) cells and subsequent lateral vessel progenitors marked by etv2.
They demonstrate that osr1 and hand2 interact antagonistically during both IM and vessel progenitor development. Further, through overexpression studies they demonstrate that osr1 is sufficient to promote differentiation of some IM cells.
Overall, the genetic studies utilize mutant models and are clean and elegant. The paper was a pleasure to read and will be a great addition to the literature.

Comments for the author

There is one major concern for the authors to address to complete the manuscript:
As this is the first analysis of the osr1 TALEN mutant in IM and vessel development, it is crucial to establish specificity of the mutation. Rescue studies with osr1 are necessary to rule out the effect(s) of other genetic alterations that may be present in the line.

Reviewer 2

Advance summary and potential significance to field

In the submitted manuscript, Perens and colleagues expand on their previous findings on the interplay of hand2 and osr1 in patterning different cell fates within the posterior lateral plate. The authors now connect osr1 function to the balanced formation of distinct endothelial progenitors and kindey precursors, potentially in a reciprocal repressor interplay with hand2.

Overall, the manuscript is well-written and features beautifully executed experiments and imaging. The lateral plate mesoderm has come back into the recent spotlight by work in several models, and the authors add a highly timely developmental nuance to the current discussion. At times, nonetheless the authors seem to over-simplify individual details that should be revisited. The manuscript and the data interpretation and presentation will benefit from rephrasing invididual statements throughout, as outlined below.

Comments for the author

Major points:
a) The introduction is overly short and would benefit from a paragraph on endothelial progenitors, given these are a main focus of the authors' work.
In particular, to the reviewer's knowledge, the authors introduce the term LVPs into a topic that is already loaded with abbreviations, so a clear introduction of these cells (i.e. later-migrating endothelial progenitors etc.) would benefit the uninitiated reader. Also, any previous work on the fate of these particular endothelial cells should be introduced and referenced.

b) The term "intermediate mesoderm" for kidney precursors is more historic than biologically correct when referring to zebrafish, as the kidney progenitors are not placed intermediate between somites and other LPM as in chick or mice. This should possibly be mentioned, and the authors have already in the past shown detailed imaging that clarifies the (often misrepresented) architecture of lateral mesoderm stripes in the zebrafish embryo.

c) The authors' conclusion that osr1 overexpression (akin to hand2 loss) suppresses LVP formation and increases kidney progenitors is an inferred fate change by (limited) marker gene expression. The authors should make clear how they interpret their data, i.e. do the LVPs turn into IM, or is there less LVPs and more IM, etc. Different possible scenarios that should be outlined.
This point comes up in the authors' speculation that a primary role of Hand2 is to inhibit IM differentiation - is this autonomous or happening by secreted factors? These different takes influence possible working models of this interesting interplay.

d) Figure 2, nuclear labeling - given these images are all the same magnification, are the nuclei in hand2; osr1 double mutants smaller throughout? Do the authors have any data that reveals cell shape and possibly cell contacts (i.e. membrane staining, etc.)?

e) Figure 3, and conceptually throughout the text - the authors seem to regard LVPs, kidney progenitors, and other lateral mesodermal cells as residing in one plane. Are these cells truly medio-laterally patterned or already also dorso-ventrally separated at the investigated time points, i.e. the red and orange cells in contact and in the same plane, with the blue cells underneath? Possibly worth discussing.

f) The authors conclude that the endoderm is not affected in osr1 mutants and present foxa2 ISH data to support this claim. While certainly taking significant effort to clarify, i.e. with whole-embryo mRNA-seq or even scRNA-seq of endoderm in osr1 mutants, the brevity of how the authors deal with the endoderm seems dismissive despite its potentially fundamental influence to the authors' conclusions. From the current data, the authors cannot exclude molecular changes in endoderm that then influence the (lateral plate) mesoderm. This issue also plays into the autonomous vs non-autonomous mode of action of osr1 and hand2 contribution to the observed phenomena.
As a more simple experiment, sox17 ISH imaged at more timepoints would further underline the authors' conclusions.

Minor points:
a) p6, stating 'osr1 is required' is possibly too strong a conclusion for the observed effect, possibly rephrase to 'osr1 influences'?

b) p7, 'posterior mesoderm' should be made more precise by stating 'posterior lateral plate mesoderm'. At other points in the text, the authors refer to 'lateral posterior mesoderm' (i.e. p8), best to homogenize this at times complex nomenclature.

c) p8, the heading 'osr1 acts in opposition to hand2 to promote intermediate mesoderm differentiation while inhibiting lateral vessel progenitor emergence' seems a tad long, recommend to shorten.

Reviewer 3

Advance summary and potential significance to field

Manuscript by Perens et al describes the analysis of osr1 zebrafish mutants which show defects vascular and kidney development. Authors demonstrate that decreased intermediate mesodermal formation correlates with premature emergence of lateral vascular progenitors, and that hand2 mutation antagonizes defects observed in osr1 mutants. They further analyze osr1 overexpression phenotypes, which inhibits LVPs while enhancing IM formation. Overall this study provides additional details regarding how osr1 and hand2 levels regulate the dimensions of kidney and vascular progenitor territories.

Comments for the author

While this study provides intriguing details regarding osr1 mutation effect on different groups of vascular progenitors, unfortunately it provides little insight into the mechanism of osr1 function. How does osr1 exert different and even opposite effects on lateral and medial vascular progenitors? Which molecular pathway does osr1 function in? Do osr1 and hand2 function in the same or different pathways? Does osr1 function in mesoderm or endoderm as previously argued by Mudumana et al., Development 2008? Furthermore, the impact of this study is somewhat reduced by an earlier study of Mudumana et al, which already demonstrated reduced kidney and expanded vascular development in osr1 deficient embryos. Although the current study provides additional details and uses genetic mutants (which largely confirm previous morpholino results), overall advance appears somewhat incremental.

Additional points.
1. It is unclear if both medial and lateral vascular progenitors differentiate prematurely in osr1 mutants or if the effect is limited to LVPs. Differentiation of medial progenitors can be assessed by the analysis of etv2 expression at 1-4-somite stages.

2. Are there more vascular endothelial cells in osr1 mutants? Are other vascular markers expanded in osr1 mutants? Is there an expansion of arterial and / or venous markers in osr1 mutants at later stages?

3. Quantification of marker expression by qPCR is needed in some experiments. In particular, quantification of gata1 expression in Fig. S3 would be helpful. Gata1 expression appears increased in hand2 mutants in Fig. S3; is this representative? Please specify how many embryos were analyzed and how many displayed the phenotypes shown.

4. The study by Mudumana et al suggested that osr1 functions in the endoderm. However, the absence of endodermal defects in osr1 mutants suggests otherwise. This is an important question that needs to be addressed. Foxa2 expression in Fig. S4 needs to be quantified better, ideally by qPCR approach. Please note in Fig. S4 how many embryos were analyzed and how many displayed the phenotypes shown. It would be helpful to supplement this with an additional marker such as sox17. Mudumana et al also showed that injection of sox32 MO in osr1 morphants rescued pronephric phenotype. Can the authors test if elimination of endoderm in osr1 mutants rescues pronephric defects?

5. Overexpression of osr1 suggests that medial vascular progenitors are expanded while LVPs are reduced.
However, it is difficult to exclude other possibilities (mislocalized position of LVPs that premature migrated for example) in the absence of specific markers. Double ISH of etv2 and other vascular endothelial or arterial markers (such as kdrl) can distinguish between medial progenitors (which are positive for kdrl and many other markers) and LVPs (which are positive for etv2 and negative for most other markers). Is expression of kdrl and other vascular markers also expanded? What is an effect on arterial and venous marker expression? Also please note how many embryos were analyzed and how many of them show the different severity phenotypes.

First revision

Author response to reviewers' comments

We are grateful to all three reviewers for their feedback regarding our work. We appreciate their view that our manuscript "*was a pleasure to read and will be a great addition to the literature*" and "*is well-written and features beautifully executed experiments and imaging*". We also value the reviewers' thoughtful suggestions for strategies to strengthen our manuscript's impact. We have now modified our manuscript to address their comments by adding new data and amending the text. Notably, our revised submission includes new additions to Figure 4, four new supplementary figures (Supplementary Figures S2, S4, S5, and S9), and a number of updates to the text, 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.

Response to Reviewer #1:

We are grateful for Reviewer #1's assessment that our studies are "*clean and elegant*" and that this work "*was a pleasure to read and will be a great addition to the literature*". Reviewer #1 also highlighted one key issue for us to address through further studies.

1. Reviewer #1 pointed out the importance of confirming that the observed phenotypes in our mutant embryos are specific consequences of the TALEN-generated mutation in *osr1*. To address this issue, we have performed the requested rescue experiments, and our revised manuscript includes new data demonstrating rescue of both the intermediate mesoderm (IM) and pronephron defects in *osr1* mutants (Fig. 4H-P, Fig. S2). Notably, we observed successful rescue using two different techniques: injection of wild-type *osr1* mRNA (Fig. S2, p. 6) and induction of expression of wild-type *osr1* via a *hsp70*-driven transgene (*hs:osr1*) (Fig. 4H-P, pp. 8-9). Together, these data provide strong evidence that the TALEN-generated mutation in *osr1* causes the observed IM and pronephron phenotypes.

   Furthermore, these rescue experiments prompted us to compare the impacts of inducing wild-type *osr1* expression in *osr1* mutants at different stages. These comparisons allowed us to define a specific timeframe during which *osr1* expression is sufficient to rescue the *osr1* mutant podocyte and pronephron tubule defects, and this information bolstered our model regarding the influence of *osr1* on IM and pronephron development. We have added this new insight to our revised manuscript (Fig. 4P, pp. 8-9).

Response to Reviewer #2:

We are grateful for Reviewer #2's assessment that our work "*features beautifully executed experiments and imaging*" and adds "*a highly timely developmental nuance to the current discussion*". Reviewer #2 also noted several aspects of our manuscript that would benefit from further elaboration. Specifically:

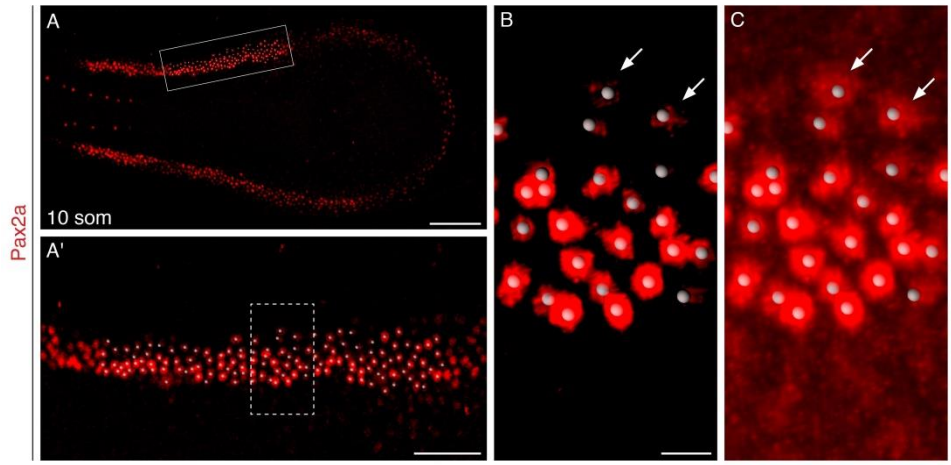
1. Reviewer #2 recommended that we add a paragraph to our introduction in order to provide more information about endothelial progenitors, especially the lateral vessel progenitors (LVPs). In particular, Reviewer #2 suggest that we include more information regarding the fate of the LVPs. We appreciate and respect Reviewer #2's request. Unfortunately, because of the 3000 word limit for a Research Report in *Development*, the length of our text is tightly constrained. We are therefore unable to add a full paragraph on endothelial progenitors to our introduction. However, we have added more background information about LVPs into two sections of our revised manuscript (pp. 3,6), including a citation to the work of Kohli and colleagues (2013) on the fate of the LVPs (p. 3).

2. Reviewer #2 requested that we clarify our definition of the term "intermediate mesoderm". As noted by the reviewer, the molecular architecture of the posterior mesoderm is different in zebrafish than in mouse or chick, and, in a prior publication (Perens et al., 2016), we described the distinct organization of the zebrafish posterior mesoderm using several markers at different stages of development. In our current manuscript, we have summarized those findings in Fig. 3A,B. Because of the length limit for a Research Report, we respectfully decline to provide a deeper discussion of the differences between zebrafish, mouse, and chick in this manuscript. Instead, we have clarified our working definition of the IM as a territory that expresses the conserved transcription factors Lhx1/Lim1 and Pax2 and contains kidney progenitors (p. 3), as these are common features of the IM across species.

3. Reviewer #2 asked us to elaborate on our interpretation of our data, with a particular emphasis on whether *osr1* influences a decision between IM and LVP fates and on whether Osr1 and/or Hand2 act cell-autonomously. In our revised discussion (p. 10), we now highlight our favored model in which *osr1* and *hand2* act cell-autonomously to direct the fate of a progenitor with the potential to contribute to the IM or LVP lineages. We also mention an

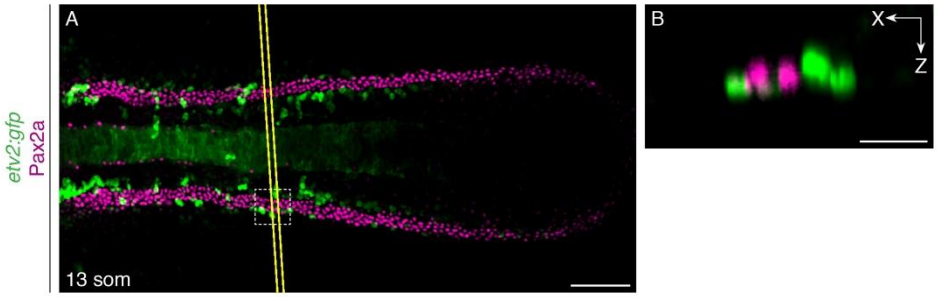
alternative model in which *osr1* and *hand2* control the production of diffusible signals that act non-autonomously to pattern the medial- lateral axis of the posterior mesoderm.

4. Reviewer #2 noted that some of the Pax2a^{+} nuclei in the *hand2*;*osr1* double mutant (Fig. 2H) appear small, suggesting the possibility of aberrant cell or tissue structure in the *hand2*;*osr1* double mutant IM. We agree that some Pax2a^{+} nuclei appear smaller than others in the *hand2*;*osr1* double mutants. We have also observed this feature in *hand2* mutants (as in Fig. 2G), including in our prior publication (see Fig. 2E in Perens et al., 2016). However, this apparent difference does not seem to be due to some nuclei being smaller than other nuclei. Instead, this difference is due to some Pax2a^{+} nuclei (usually those on the lateral edge of the IM) having a smaller maximal area of brightness when we decrease the image brightness so that background fluorescence is no longer visible, as discussed in our Materials and Methods section (p. 13). Importantly, if we increase image brightness, the full morphology of the nuclei becomes visible, and the nuclei that appeared small are revealed to be comparable in size to other Pax2a^{+} nuclei (see Reviewer Figure R1, below). We should also note that we did not examine cell shape or cell contacts in these experiments, as our primary goal was to assess the number of Pax2a^{+} cells and not the physical architecture of the tissue.



Reviewer Figure R1.
(A-C) Dorsal views, anterior to the left, show three-dimensional reconstructions of Pax2a immunofluorescence in the IM of a *hand2*;*osr1* double mutant embryo at 10 som. (A') Magnification of boxed 250 μm long region used for counting Pax2a^{+} cells. White dots indicate Pax2a^{+} nuclei. Images are from Fig. 2H and Fig. 2H'. (B) Magnification of boxed region in (A'). Examples of Pax2a^{+} nuclei that appear small are indicated with arrows. (C) Version of the image in (B) in which brightness has been increased. Nuclei that appear small in (B), such as the examples marked by arrows, appear comparable in size to other Pax2a^{+} nuclei when brightness is intensified. Scale bars: 100 μm (A), 50 μm (A'), 10 μm (B).

5. Reviewer #2 asked us to clarify the dorsal-ventral arrangement of the cells in the lateral posterior mesoderm. As noted by the reviewer, we have portrayed the LVPs, the IM, and other lateral posterior mesodermal cell types as residing in a single plane. At the timepoints that we examine here, it is indeed our impression that these cells are found in roughly the same dorsal-ventral plane. As an example, Reviewer Figure R2 (below) provides a transverse section view of the embryo from Fig. 4C, showing that the Pax2a^{+} cells and the *etv2:gfp*$^{+}$ cells (both medial and lateral vessel progenitors) all reside in roughly the same plane along the Z axis.



Reviewer Figure R2.
(A,B) Three-dimensional reconstruction of Pax2a and GFP immunofluorescence in a wild-type embryo carrying *Tg(etv2:egfp)* at 13 som. (A) Dorsal view, anterior to the left; image is from Fig. 4C. (B) Transverse view (XZ) of a 10 μm section at the location of the boxed region in A. Scale bars: 100 μm (A), 30 μm (B).

6. Reviewer #2 suggested that we expand our assessment of the endoderm in *osr1* mutants and requested that we, at a minimum, examine expression of *sox17*. We appreciate the value of extending this aspect of our analysis, and we now include new data regarding the endoderm in our revised manuscript.

   First, we assessed *sox17* expression in *osr1* mutants, taking special care to precisely stage the embryos in order to minimize the variability that can result from the rapid migration of endodermal cells during shield stage. By taking this rigorous approach to staging (which was admittedly more precise than the approach taken in our earlier analysis of *foxa2* expression), we were able to observe a trend toward a higher density of *sox17*-expressing endodermal progenitors at the blastoderm margin of *osr1* mutants. These new results are reported in our revised manuscript (Fig. S9A,B), and we note that these results are similar to the increase in endodermal progenitors previously observed in *osr1* morphants (Mudumana et al, 2008). However, although endodermal expression of *foxa2* was reported to be increased in *osr1* morphants at 18 som (Mudumana et al., 2008), we did not observe any consistent difference in the *foxa2*-expressing endoderm when comparing wild-type (n=61) and *osr1* mutant (n=19) embryos from three independent clutches at that stage. These data are also reported in our revised manuscript (Fig. S9C,D).

   Second, since prior studies had shown that disruption of endoderm formation via *sox32* knockdown could partially rescue pronephron tubule defects in *osr1* morphants (Mudumana et al., 2008; Tomar et al., 2014), we examined the effects of injecting a *sox32* morpholino (MO) into *osr1* mutants. We found that *sox32* knockdown broadened the appearance of the IM in both wild-type and *osr1* mutant embryos, compared to the IM of uninjected siblings. These new results are reported in our revised manuscript (Fig. S9E-H). The broadened morphology of the IM in *sox32* morphants was distinct from the appearance of the enlarged IM in embryos overexpressing *osr1* (Fig. 4F), although this broadened morphology did remind us of the morphogenetic defects observed for multiple mesodermal derivatives, including the pronephron, blood, myocardium, and vasculature, in the *sox32* mutant *casanova* (Alexander et al., 1999). Furthermore, while we found that induction of wild-type *osr1* expression could rescue the appearance of the *osr1* mutant IM (Fig. S2D, Fig. 4K), we could not reach the same conclusion in the context of *sox32* knockdown, since the morphology of the IM was similarly aberrant in both the wild-type and *osr1* mutant embryos that were injected with the *sox32* MO (Fig. S9G,H).

   Nevertheless, our observation of increased formation of endoderm progenitors in *osr1* mutants at shield stage suggested a possible influence of excess endoderm on IM and pronephron development, and we sought to address this with a set of new experiments reported in our revised manuscript (Fig. 4H-P). Specifically, we chose to assess when induction of *osr1* expression is able to rescue the *osr1* mutant defects. We found that induction of *osr1* expression at tailbud clearly rescued the *osr1* mutant IM, podocyte, and pronephron tubule defects (Fig. 4H-P). Thus, *osr1* function after gastrulation is sufficient to

regulate IM development, and *osr1* function during earlier stages of mesendoderm development is not absolutely required for proper IM and pronephron formation. Conversely, induction of *osr1* at 10 som failed to rescue the pronephron defects in *osr1* mutants (Fig. 4P). Together, our analyses suggest a time window after the completion of gastrulation during which *osr1* function is sufficient to promote the development of pronephron progenitors within the IM. Intriguingly, the timepoint at which *osr1* induction was no longer able to rescue pronephron development coincides with the normal timing of LVP emergence. These new insights are reported in our revised manuscript (pp. 8-9).

7. Reviewer #2 suggested that we avoid use of "required" in our statement "*osr1* is required to constrain vessel progenitor development…" on p. 6. As requested, we have removed "required" from this statement in our revised manuscript (p. 6).

8. Reviewer #2 suggested that we replace the phrase "posterior mesoderm" with the phrase "posterior lateral plate mesoderm" when discussing patterns of *hand2* and *osr1* expression on p. 7. We greatly respect the reviewer's interest in homogenizing the complexity of the nomenclature used in the field; nevertheless, we prefer to avoid the phrase "lateral plate" in this manuscript, precisely because it is used in very different ways throughout the literature. Here, we have opted to use the phrase “posterior mesoderm” simply to denote that we are describing the expression patterns in the posterior mesoderm and not in the anterior mesoderm, where the patterns are quite different.

9. Reviewer #2 suggested that we shorten the section heading on p. 9. In our revised manuscript, we have simplified this heading through the use of abbreviations (p. 9).

Response to Reviewer #3:

We are grateful for Reviewer #3's acknowledgement that our work “*provides additional details regarding how osr1 and hand2 levels regulate the dimensions of kidney and vascular progenitor territories*”. Reviewer #3 also expressed interest in a number of open questions regarding the precise mechanism of *osr1* function. We agree that these open questions are intriguing ones for future investigation, although we consider them to be beyond the scope of the current study. Finally, Reviewer #3 expressed the view that the advance provided by our manuscript "*appears somewhat incremental*", especially in light of previous work from Mudamana and colleagues (2008). We respectfully disagree, as our point of view is more aligned with the positive perspective presented by Reviewer #1 and Reviewer #2. As noted in our manuscript, our study provides several meaningful advances beyond the results presented by Mudamana and colleagues (2008). First, this prior study of *osr1* morphants argued that *osr1* is not required for the initial formation of the IM, whereas our data reveal an early defect in IM formation in *osr1* mutants (Fig. 2, Fig. 4, and Fig. S2). Second, while Mudamana and colleagues (2008) reported an expansion of the vessel progenitors located at the anterior extent of the progenitor territory in the posterior mesoderm, they did not examine the LVPs that arise at the boundary between the intermediate mesoderm and the *osr1*-expressing mesoderm. Our analysis of the LVPs in *osr1* mutants has revealed a previously unappreciated role of *osr1* in controlling the timing of LVP differentiation (Fig. 3). Third, our revised manuscript features new data that directly address a fundamental question regarding the early phases of kidney development: when does *osr1* function to promote kidney formation? Through our transgenic rescue studies, we have now delineated the timeframe during which *osr1* acts to promote podocyte and pronephron tubule development (Fig. 4). Taken together, we feel that these studies meaningfully advance our understanding of the roles of *osr1*.

1. Reviewer #3 asked whether the medial vessel progenitors, like the lateral vessel progenitors, differentiate prematurely in *osr1* mutants. We appreciate the suggestion to include data addressing this point. In our revised manuscript, we present new data demonstrating that the timing of the appearance of the medial vessel progenitors is unaffected in *osr1* mutants (Fig. S4).

2. Reviewer #3 wondered whether the premature differentiation of LVPs in *osr1* mutants ultimately yields a larger vasculature or an expansion of specific vascular markers. We appreciate the suggestion to include information on these aspects of the *osr1* mutant

phenotype. In our revised manuscript, we present the expression patterns of two vascular markers – *flt4* and *mrc1a* – in *osr1* mutants (Fig. S5). In our previous work, we had demonstrated that *hand2* mutants, which fail to form LVPs, lack expression of *flt4* and *mrc1a* within the cardinal vein (Perens et al., 2016). In contrast, expression of these genes is not affected in *osr1* mutants (Fig. S5E,H). While we cannot rule out changes to the total number of vascular cells or alterations in other parts of the vasculature, it seems that the premature differentiation of LVPs in *osr1* mutants does not lead to alterations in the specification of the dorsal aorta or posterior cardinal vein.

3. Reviewer #3 raised concern about the relative expression levels of *gata1* in *hand2* mutants compared to wild-type embryos. We apologize for the confusion caused by our choice of wild-type embryo to display in the original figure. As we have previously reported, there is no evident alteration of *gata1* expression in *hand2* mutants (Perens et al., 2016). However, the wild-type image selected for our original figure was not a particularly representative example of the wild-type expression intensity. In our revised manuscript, we have included a more representative image of wild-type *gata1* expression (Fig. S6A) that demonstrates comparable expression to that seen in *hand2* mutants. In addition, to confirm the lack of a *gata1* phenotype in *hand2* mutants, we examined embryos from incrosses of *hand2* heterozygotes and observed no evident phenotypes (total n=43; *hand2* mutants=8).

4. Like Reviewer #2, Reviewer #3 requested a more detailed assessment of the role of *osr1* in endoderm development and how that role might impact pronephron development. As noted above, we appreciate the value of extending this aspect of our analysis, and we now include a series of new data regarding the endoderm in our revised manuscript. Please see our response above to Reviewer #2, Comment #6, for a full description of the new experiments performed and the new data reported in our revised manuscript (Fig. 4, Fig. S9, pp. 8-9).

5. Reviewer #3 requested a more complete assessment of the vessel progenitors and vasculature when *osr1* is overexpressed. We agree with the reviewer that it is challenging to evaluate the different populations of vascular progenitors within the posterior mesoderm that are regulated in different manners by *osr1*. As the reviewer also noted, while *etv2* is expressed in multiple populations of these territories containing vascular progenitors, *kdrl/flk1* is only expressed in a subset of the proximal and medial vascular progenitors. In response to the reviewer's requests, we have taken several approaches to a more thorough evaluation of the vascular phenotypes in embryos overexpressing *osr1*.

   First, as requested by Reviewer #3, we have included quantification of the numbers of embryos observed in each of the phenotypic categories shown in Fig. S7. In coordination with this, we have replaced the image shown in Fig. 4B with one that is representative of the category containing a plurality of the examined embryos; the full range of phenotypic categories is still shown in Fig. S7.

   Second, as requested by Reviewer #3, we have examined *flk1* expression in both the *osr1* loss-of-function and gain-of-function scenarios, and these data are reported in our revised manuscript (Fig. S5A-C). In the *osr1* mutant, *flk1* expression, like *etv2* expression, is expanded in the proximal territory but appears unaffected in the medial territory (Fig. S5B); this phenotype was observed in 82% of *osr1* mutants (n=17) and in 0% of wild-type embryos (n=43). In embryos overexpressing *osr1*, we observed a mild increase in *flk1* expression in the medial territory (Fig. S5C); this phenotype was observed in 20% of *hs:osr1* embryos (n=56). The observed increase in *flk1* expression may correspond to an expansion of the population of medial vessel progenitors in embryos overexpressing *osr1*.

   Third, as requested by Reviewer #3, we evaluated *flt4* and *mrc1a* expression in embryos overexpressing *osr1*, and these data are reported in our revised manuscript (Fig. S5D,F,G,I). Expression of both genes was increased in all *hs:osr1* embryos examined (Fig. S5F,I), including areas of ectopic expression within the trunk (ectopic *flt4* in 59% of *hs:osr1* embryos, n=52; ectopic *mrc1a* in 14% of *hs:osr1* embryos, n=22).

   Together, these analyses provide more depth to our understanding of the impact of *osr1*

overexpression on the vasculature. However, we note that these data do not definitively resolve the still-open question of the identity of the excess *etv2*- expressing cells found in the medial territory of embryos overexpressing *osr1*.
Further characterization of these cells will be a valuable topic for future studies, beyond the scope of our current manuscript.

Second decision letter

MS ID#: DEVELOP/2020/198408

MS TITLE: osr1 couples intermediate mesoderm cell fate with temporal dynamics of vessel progenitor cell differentiation

AUTHORS: Elliot Perens, Jessyka Diaz, Agathe Quesnel, Amjad Askary, Gage Crump, and Deborah Yelon

I have now received all the referees reports on the above manuscript. 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 positive and the referees only have relatively minor issues for you to address prior to publication. One of the referees suggests the manuscript would be improved if you change from the research report to article format - I will leave it to you to decide on this issue. 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.

Reviewer 1

Advance summary and potential significance to field

The authors have provided important insights into the roles of the transcription factor osr1 during intermediate mesoderm development. This information is essential to uncovering the gene regulatory networks that control early development and has value for understanding the basis of congenital defects.

Comments for the author

I recommend acceptance of the manuscript.

Reviewer 2

Advance summary and potential significance to field

The authors added more data to their previous submission and addressed several comments by the three reviewers. Overall, the manuscript adds interesting insights to the still unclear biology of how key mesodermal fates distinguish from each other during early development. The reviewer would have much hoped the authors would have chosen a longer format for their manuscript to provide more context and room for their phenotype descriptions, as the short form of the work is overly complex and brief at times. As it stands now, the manuscript funds on documenting genetic interactions without mechanistic insight into how osr1 and hand2 act with/against each other. Nonetheless, the data provide a starting point for a deeper mechanistic dive in future work.

Comments for the author

Remaining points:
1) the authors are encouraged to incorporate the reviewer figures into supplementary figures to have these documented (given no review summary file will be provided by Development?).

2) The used nomenclature of cell types should be revisited to ensure homogenous use throughout the text.

3) The conclusion that Osr1 and Hand2 act autonomously OR non-autonomously is highly ambiguous given that these are the only two options for their action. Is there any scenario the authors favor based on any of the presented data?

4) The transition between the data presented on page 5 and the next heading "osr1 is required to generate the full complement of intermediate mesoderm" is overly abrupt and might benefit from a concluding or bridging sentence.

Reviewer 3

Advance summary and potential significance to field

Manuscript by Perens et al describes the analysis of osr1 zebrafish mutants which show defects vascular and kidney development. Authors demonstrate that decreased intermediate mesodermal formation correlates with premature emergence of lateral vascular progenitors, and that hand2 mutation antagonizes defects observed in osr1 mutants. They further analyze osr1 overexpression phenotypes, which inhibits LVPs while enhancing IM formation. Overall this study provides significant insight regarding how osr1 and hand2 levels regulate the dimensions of kidney and vascular progenitor territories.

Comments for the author

In the revision, the authors provided additional data which have largely addressed most of my previous concerns. My only remaining suggestion is regarding presentation and interpretation of the endodermal defects observed in osr1 mutants, as listed below.
1. Currently data shown in Fig. S9 are only briefly mentioned in the text. It would be helpful to describe these results in the main text more completely.

2. The authors suggest that endoderm is unlikely to mediate observed vascular and IM defects because osr1 expression can rescue these defects after the tailbud stage. However, this experiment addresses the timing of osr1 function and not its tissue-specific requirement. Is it possible that osr1 expression after tailbud stage alleviated endodermal defects which then affected vasculature / IM? A broader discussion or at least an acknowledgement of alternative explanations is warranted.

Second revision

Author response to reviewers' comments

We are grateful to all three reviewers for their positive feedback regarding our revised manuscript. We are pleased that the reviewers were generally satisfied with our response to their initial reviews, and we value their additional input regarding our work. We also appreciate being given editorial permission to exceed the standard word limit for a Research Report, and we have utilized this extra space to respond to the reviewers' comments. We have modified our revised manuscript to address their requests, and our updates to the text are highlighted in the attached document. Our point-by-point responses to the reviews are assembled below.

Response to Reviewer #1:

We are grateful for Reviewer #1's recommendation for acceptance of the manuscript.

Response to Reviewer #2:

We are grateful for Reviewer #2's acknowledgement that our revised manuscript "*added more data*", "*addressed several comments by the three reviewers*", and "*adds interesting insights to the still unclear biology of how key mesodermal fates distinguish from each other during early development*." Reviewer #2 also expressed the concern that "*the short form of the work is overly complex and brief at times*" and expressed the desire for "*a longer format*". In order to address this issue, we are pleased to have received editorial permission to exceed the standard word limit for a Research Report. Using this extra space when appropriate, we have addressed the feedback from Reviewer #2 as follows:

1. Reviewer #2 encouraged us to add the two figures that we included in our previous response to the reviewers as supplementary figures. We note that these figures will be included in the "Peer review history" provided on the *Development* website. With this in mind, we respectfully decline to include these as supplementary figures, since we feel that "Peer review history" format will be the most effective way to present this information within the context of Reviewer #2's specific questions.

2. Reviewer #2 recommended refinement of the cell type nomenclature used throughout the text. In response to this request, we have homogenized the terminology used to describe the different territories of the posterior mesoderm. Notably, we have removed or revised the terms "posterior lateral mesoderm" (p. 2), "lateral mesoderm" (pp. 3, 9), and "lateral posterior mesoderm" (pp. 3, 4, 22). In addition to the general term "posterior mesoderm", we use the following terms to describe territories within the posterior mesoderm:
   - Intermediate mesoderm (IM): Introduced on p. 3 as the location of kidney progenitors and defined by the expression of Lhx1/Lim1 and Pax2. Illustration of the IM in the context of the zebrafish posterior mesoderm is shown in Fig. 3A,B.
   - Kidney progenitor territory/kidney progenitors: Broadly defined as any cells that contribute to the pronephron and introduced on p. 3 as being within the IM.
   - Vessel progenitor territory/vessel progenitors: Broadly defined as any cells that give rise to vascular endothelial cells. Three subdomains of vessel progenitors within the posterior mesoderm – lateral, medial and proximal – are defined below. These subdomains are illustrated in Fig. 3A,B and represented in Fig. 3C,D,F-I by their expression of *etv2* (arrows indicating lateral subdomain, arrowheads indicating medial subdomain, and asterisks indicating proximal subdomain).
   - Lateral vessel progenitors (LVPs): Introduced on p. 3 as stripes of vessel progenitors arising between the IM and the *osr1*-expressing territory, and further described on p. 3 as cells that contribute to the cardinal vein (Kohli et al., 2013).
   - Medial vessel progenitors: Defined on p. 6 as vessel progenitors located medial to the IM and noted to arise earlier than LVPs (Kohli et al., 2013; Perens et al., 2016).
   - Proximal vessel progenitors: Introduced in Supplementary Figure S3 as *etv2*-expressing cells in the most proximal portion of the posterior mesoderm.
   - Blood progenitors: Introduced on p.7 and in Supplementary Figure S6 as *gata1*-expressing cells, and illustrated in Fig. 3A,B as being medial to the IM.

3. Reviewer #2 suggested that we add further discussion regarding the cell autonomy of *osr1* and *hand2* function. In response to this request, we have added text to our revised manuscript (p. 10) to discuss why we favor a cell-autonomous model and to comment on the future experiments needed to address autonomy. Briefly, we favor a model in which *hand2* functions cell-autonomously, because of the appearance of ectopic Pax2a-expressing cells within *hand2*-expressing cells in *hand2* mutants (Fig. S8C; Perens et al., 2016). Furthermore, we propose that *osr1* acts in the same manner as *hand2*. In addition to being expressed in the same territory (Fig. 3J-O; Perens et al., 2016), *osr1* and *hand2* seem to function in the same timeframe: the stage after which induction of *osr1* expression fails to rescue the *osr1* mutant pronephron defects (Fig. 4P) coincides with the stage after which *hand2* overexpression fails to inhibit pronephron development (Perens et al., 2016). Even though we favor a cell-

autonomous model, we also discuss the alternative possibility that *osr1* may function cell non-autonomously, and we present the need for future mosaic analysis to resolve this issue.

4. Reviewer #2 recommended that we modify the transition between the first and second subsections of our Results and Discussion section. As suggested, we have revised the end of the first subsection (p. 5) and the beginning of the second subsection (p. 6) to improve this transition.

Response to Reviewer #3:

We are grateful for Reviewer #3's acknowledgement that our revised manuscript "*provided additional data which have largely addressed most of my previous concerns*." Reviewer #3 also requested that we elaborate on our coverage of the endodermal defects in *osr1* mutants, as follows:

1. Reviewer #3 suggested that the main text of our manuscript should include a more thorough description of the endodermal defects observed in *osr1* mutants, shown in Supplementary Figure S9. As requested, we have added text describing these results to our revised manuscript (p. 8).

2. Reviewer #3 noted the implication in our revised manuscript that the results of our temporal rescue experiments make it unlikely that the *osr1* mutant endoderm mediates the observed vascular and IM defects. Additionally, Reviewer #3 pointed out that it is still possible that overexpression of *osr1* after the tailbud stage could potentially alleviate the endodermal defects in *osr1* mutants. With this in mind, Reviewer #3 requested that we provide additional discussion to acknowledge this alternative interpretation. As suggested, we have added text to our revised manuscript (p. 10) to discuss this topic. Specifically, while we state that our temporal rescue experiments argue against a mechanism in which *osr1* regulates pronephron development by controlling the initial formation of endoderm progenitors, we also note that we cannot rule out a later role for *osr1* in the endoderm.

Third decision letter

MS ID#: DEVELOP/2020/198408

MS TITLE: osr1 couples intermediate mesoderm cell fate with temporal dynamics of vessel progenitor cell differentiation

AUTHORS: Elliot Perens, Jessyka Diaz, Agathe Quesnel, Amjad Askary, Gage Crump, and Deborah Yelon
ARTICLE TYPE: Research Report

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