Spatiotemporal sequence of mesoderm and endoderm lineage segregation during mouse gastrulation

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MS TITLE: Spatiotemporal sequence of mesoderm and endoderm lineage segregation during mouse gastrulation

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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. The referees recognise you are addressing an important issue and one that has been contentious between the developmental biology and stem cell biology field. However, as both Referee 2 and 3 point out, you document a population of Foxa2/Mesp1 expressing cells that could represent a transient bipotential population of mesendoderm precursors. It seems crucial to investigate this possibility. Better quantification of imaging data, with a focus on the double positive cells, could address this issue. Additionally, extending the computational analysis of the single cell data with, for example, pseudotime analysis and a more thorough examination of the Foxa2/Mesp1+ cell population at the PS-DE-AM branch point would also strengthen the study.

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.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater

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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

The manuscript by Probst et al. tackles the question of the existence of a mesendodermal progenitor in mouse embryo gastrulation. It comes tho the conclusion that these cell lineages are specified within the primitive streak, before cell delamination, and that mesoderm and endoderm are generated from spatially and temporally distinct progenitors.
This conclusion is in agreement with punctual data from other reports, but it had never been formally shown before. Here the authors use elegant lineage tracing studies with novel reporters, and careful single cell transcriptome analysis, to highlight the spatial and temporal appearance of each population.
It is a very useful study for the field, carefully documented, with high quality imaging, ans smart use of novel and published sets of single cell RNASeq data.

Comments for the author

The data obviously raise many additional questions, but my assessment is that the authors properly respond to their specific research question and that additional experiments aren't necessary. However, from the current data, some additional quantitative analysis might be interesting.

The current model for definitive endoderm morphogenesis is that cells undergo a EMT followed by MET. It would add to the discussion to propose an opinion on how those current data may give more insight into that model, perhaps by looking at genes associated with EMT in the distinct populations. Similarly, some authors suggest that endoderm cells may be passively dragged by mesoderm migration perhaps as small groups, so looking for genes associated with migration might help. Moreover, from the stained embryos, the authors might be able to estimate the morphology of mesoderm versus endoderm fated cells, as well as their probability to be surrounded by neighbours of a similar fate.

Asa minor comment, and although the paper is very well constructed and written, I would suggest to perhaps try and be a bit more concise and more precisely separate the specific conclusions from the different approaches as it feels slightly repetitive at times.

Reviewer 2

Advance summary and potential significance to field

Probst et al use a combination of imaging of reporter lines, immunohistochemistry, and single cell transcriptomics to argue for that Eomes positive progenitors in the epiblast give rise to independent lineage specified progenitors for endoderm and anterior mesoderm in a spatial and temporally distinct manner. They employ a set of reporters (Eomes-membrane Tomato/ nuclear GFP and Mesp1-membrane Venus), their own limited single transcriptomics, FoxA2 antibody staining, a CreERT-Eomes allele and re-analysis of a larger transcriptomic dataset from Bertie Göttgens lab. For the transcriptomics analysis they employ some interesting new tools including RaceID and FateID, in addition to generating a useful new reporter mouse line, the Mesp1-membrane Venus.

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The fundamental observation contained in this paper, that AM and DE arise independently is a nice confirmation of the clonal analysis from Val Wilson's and previous fate maps. As they have not considered the axial mesoderm and endoderm, it is hard to make a strong conclusion about the absence of a common precursor of mesoderm and endoderm outside of the epiblast, so their conclusions are relatively limited. If this were the only issue, perhaps one could consider publication in Development. However, there are a number of additional issues with the manuscript that make it difficult to evaluate their conclusions. Thus, their conclusions about Eomes uniformity, FOXA2 expression, Mesp1/FOXA2 co-expression are based on individual images without any quantification. In addition, the single cell transcriptomics is relatively superficial. The manuscript contains lots of tSNEs, but little in depth analysis and incomplete cluster annotation. The authors wish to make a conclusion about progenitor specification, using sophisticated reporters, but with a complete absence of live imaging. Finally there are number of confusing and sweeping statements that are lacking in significant substantiation. For all these reasons, this manuscript would seem too preliminary for publication or consideration in a reasonable period of revision.

Comments for the author

Specific issues found in the text. This is not exhaustive, but meant to point out the methodological issues with the study as it currently stands.

1. The writing and abbreviations are confusing. Some abbreviations are not defined.
2. In Fig 1, they localize mesoderm and endoderm progenitors, but do not explain how these are defined.
3. On line 136 the authors state that all cells leaving the primitive streak are Eomes positive. However, in the referenced figures, they show no quantification and immunohistochemistry is based on single example sections only. Better quantification is required for this conclusion.
4. On line 140, they state there are some reporter negative cells in the endoderm, and state these are “most likely,” VE cells that lose reporter expression. However, no demonstration that these are VE cells has been presented. They also maintain elsewhere that Eomes is expressed throughout the VE, does this mean it is down regulated in the VE more rapidly than the DE?
5. The color scheme that the authors have chosen for the immunohistochemistry is very difficult to interpret.
6. In supplementary Fig 1A and B, there are clusters that appear composed of one or two cells (or even no cells).
7. In Fig 1I and L they did not assign the cluster identity for all the clusters. In L, there are clear clusters that were not assigned and it is not clear why these are merely defined as “other.”
8. There number of clusters defined in the supplemental data are different from those defined in the main figures.
9. On line 175, they say there is no sub-cluster within the E6.75 epiblast, but they show sub-clustering of epiblast in S1.
10. They have introduced a bias by hand picking cells at E6.5, but using FACS for E7.5. Why introduce this bias? Why not included FACS sorted cells at E6.5 to control for this bias? We imagine that the different experiment approaches here would introduce technical batch variation. Is this why they have not analyzed their data together? Combining these stages would seem important, as progenitors are specified across a window of developmental time.
11. From line 191 they claim embryonic Eomes positive cells at E6.75 are quite “similar,” to each other. However, there is no correlation data to support this. Nor is Eomes included in the heat maps they generated. In 1K, Eomes expression appears quite heterogeneous.
12. On line 208 they claim they identify the earliest Eomes dependent mesoderm at E6.5, but its hard to fathom how they can make this claim in the absence of either live imaging or extensive quantification. A similar statement is made on line 212.
13. On line 220, they also make statements about numbers of Mesp1 negative cells, but its hard to understand how they can make this conclusion based on the data in Fig 2I and L.
14. At E6.5, line 246, they claim that FOXA2 expressing cells are DE progenitors. How do they know? FOXA2 marks a spectrum of lineages including axial mesoderm and floor plate.
15. In Fig 3B they point to a region of the embryo and say it is DE. Why?
16. Around line 256, they claim that there are only “rare,” Mesp1/FOXA2 double positive cells. However, in the single transcriptomics in Fig S4, the double positive population appears quite extensive. Does this represent a discrepancy? They claim these could be progenitors of the cardiac ventricles, but there is no data to support this. Furthermore, on line 260, they dismiss their identity

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as a bipotent progenitor population without providing any data and based on their position at the junction of two domains of expression.

17. In Fig 4, they claim to harvest embryos from E6.25-7.5, culture for 90 minutes with 4OHT and then culture for 24 hours. How did they collect the samples for E6.5? Where are the embryos cultured for 24 hours from E7.5? The methodology is very unclear.

18. As they have clear defined beginning and end points for their analysis, it is not clear why they have not generated pseudotime on the transcriptomic data, so that their work could be benchmarked against the analysis coming from other groups.

19. On line 289 they say that upto 5% of the cells could be double positive for Mesp1/FOXA2. This would seem a reasonable percentage of cells for a progenitor population.

20. While Mesp1 and FOXA2 are good markers, one wonders why they can't exploit all of this transcriptomic data to find additional markers that could be used to strengthen any conclusions.

21. The application, FateID, is not explained. It would appear just a variation on pseudotime. The target population in Fig 5I, J appears to have a significant set of overlapping cells. If this is not the case, then these target populations need to be better represented. They also appear to pool cells from E6.5/E6.75, but not other stages. Is this a good idea?

22. What is the meaning of the idea that Eomes single positive cells are biased in a particular direction? Is this based just on gene expression similarities? They dismiss the notion that these trajectories could represent distinct progenitors, based on the differential gene expression not revealing any known markers of either lineage, but perhaps there are new markers in their dataset.

23. There is a good FOXA2-Venus reporter, why have the authors not used this. It is a fusion protein and therefore largely nuclear.

24. In the discussion they say that almost all cells leaving the primitive streak are Eomes positive. In the results, it is all cells. It would seem difficult to conclude all cells express Eomes based on the data in S3. What then are the Eomes negative cells? As mentioned in the earlier comment, it is also difficult to assess how the cluster were assigned and how Eomes is expressed across each of these clusters.

25. In multiple figures they discuss coexpression and correlation, but fail to produce a regression line or correlation score. See for example, Fig 3N.

Reviewer 3

Advance summary and potential significance to field

The study by Probst et al addressed the molecular and cellular basis of one of the earliest cell fate decisions in the mammalian embryos, the segregation of the endoderm (DE) and mesoderm (AM) lineages during gastrulation. This is an important issue with several unresolved questions that the authors address.

Using high resolution imaging, genetic lineage tracing and single cell transcriptomics they examine the emergence of the AM and DE from Eomes-expressing cells in the primitive streak. In addition to rigorously confirming previous observations on the temporal-spatial dynamics of DE vs AM. Specifically they examine whether or not these two lineage emerge from a common "mesendoderm progenitor" which has been postulated mostly from in vitro differentiation of pluripotent stem cells. In general the quality of the data is excellent, particularly the embryo staining and lineage tracing. However I have some reservations about the authors conclusions and with their assumption that the common progenitor would have to be a large population of cells that co-express Foxa2 and Mesp1. I strongly suggest that the authors include a more balanced interpretation of the data and provide additional analysis that would reflect the alternative hypothesis. If the authors can address my concerns then I feel that this will be a valuable contribution to the field.

Comments for the author

Major

1. The main conclusion that they don't find any evidence of a Foxa2/Mesp1 co-expressing cell population is simply not true. Their analysis of the Pijuan-Sala data (which has sufficient cells) show that 2-5% of the cells are Foxa2/Mesp1 double positive. The authors explain this away with the statement,

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“Their location only at the border of Mesp1 and Foxa2 positive areas suggests that these are specialized cells rather than bipotential progenitors.” While this is possible, the data also support an alternative interpretation, where the bipotential progenitor is very transient with the respective DE and AM GRNs mutually repressing each other rapidly. This would be equally interesting. I suggest presenting the data and both interpretations.

2. In Fig. 5 the authors should include a separate UMAP plot showing Foxa2/Mesp1 double positive cells. In Fig 5G-J there is a population of double positive cells at the DE-AM branch point that appears to be biased to both Mesp1 and Foxa2 fates. Indeed the authors say ..” The cells closer to the branching point mostly did not display a clear fate bias showing an intermediate probability for both lineages”. This is precisely the predicted behavior of the common progenitor, so I don't understand why the authors dismiss this interpretation.

3. The assumption that a common biopotential progenitor would form a separate cluster is a bit of a stretch. It might, but it might also simply fall in a continuum of cell states between PS DE and AM, which is what my interpretation of the data show. In this case the Eomes-expressing PS cells are the common progenitor and as cells ingress through the streak they very transiently express markers of both lineages as they segregate.

Minor
4. In Fig.4 the authors should include the EmM scoring data in the Fig 4E histogram. This would help directly comparing DE vs Mes contribution over time.

5. In the discussion a brief consideration of the support for a common progenitor from PSC differentiation literature would be helpful. Perhaps an artifact of in vitro, or perhaps the cultures allow one to isolate the transient nature of cells in the primitive streak.

6. The authors need to more clearly acknowledge the limitations of their own scRNA-seq data set. While manual isolation of the cells is an impressive example of embryology, the fact that they have less than 20 DE and AM cells makes it really impossible to draw robust conclusions considering the transcript dropout. Of course they mostly use the Pijuan-Sala data, which is appropriate, but they should acknowledge this more directly.

7. Although I am versed in both the embryology and scRNA-seq applications, I cannot assess the actually computational work and recommend a reviewer with expertise in that area. In general the robustness of the scRNA-seq analysis would be enhanced if alternative methods of trajectory inference, such as Monocle or URD showed similar results.

First revision

Author response to reviewers' comments

Reviewer 1

Advance Summary and Potential Significance to Field: The manuscript by Probst et al. tackles the question of the existence of a mesendodermal progenitor in mouse embryo gastrulation. It comes to the conclusion that these cell lineages are specified within the primitive streak, before cell delamination, and that mesoderm and endoderm are generated from spatially and temporally distinct progenitors. This conclusion is in agreement with punctual data from other reports, but it had never been formally shown before. Here the authors use elegant lineage tracing studies with novel reporters, and careful single cell transcriptome analysis, to highlight the spatial and temporal appearance of each population. It is a very useful study for the field, carefully documented, with high quality imaging, and smart use of novel and published sets of single cell RNASeq data.

The data obviously raise many additional questions, but my assessment is that the authors properly respond to their specific research question and that additional experiments aren't necessary.

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However, from the current data, some additional quantitative analysis might be interesting. The current model for definitive endoderm morphogenesis is that cells undergo an EMT followed by MET. It would add to the discussion to propose an opinion on how those current data may give more insight into that model, perhaps by looking at genes associated with EMT in the distinct populations. Similarly, some authors suggest that endoderm cells may be passively dragged by mesoderm migration, perhaps as small groups, so looking for genes associated with migration might help. Moreover, from the stained embryos, the authors might be able to estimate the morphology of mesoderm versus endoderm fated cells, as well as their probability to be surrounded by neighbours of a similar fate.

These suggestions the reviewer is bringing up are very interesting questions to investigate. We have now included the expression analysis of EMT and migration markers in the Eomes positive cell populations (Fig.5D, E). Interestingly, we find that both EMT and migration genes are expressed at lower levels in the Foxa2/Eomes double positive population than in the Mesp1/Eomes double positive populations. So even though DE progenitors delaminate from the epiblast, it seems that the EMT they undergo might be less complete. The fact that migration genes are also less expressed in Foxa2/Eomes double positive DE progenitors supports the idea that DE cells could be dragged along in the mesoderm wings. We have included a section in the main text and heat maps and violin plots to Figure 5, discussing these results (Fig. 5D, E and lines 361 - 368).
Unfortunately, in our imaging approach we are unable to assess morphological differences of AM vs DE progenitors, due to reporter-mediated membrane staining of AM cells and FOXA2 nuclear staining in DE progenitors. However, we re-analysed sections of Mesp1/Foxa2 stained embryos, now focusing on the distribution of AM and DE cells. At E6.5/E6.75 both populations are still located relatively closely to the PS and there is some intermingling at the intermediate level PS, where the two domains meet. Here, the two cells types seem to be more or less randomly mixed. At E7.25 we still find mixing of mesoderm and DE progenitors in the mid-region of the PS, and we additionally find previously described FOXA2 positive DE cells within the mesoderm layer (Viotti et al, 2014). DE cells within the mesoderm layer are either single cells or are seen in small groups of 2-4 cells. These small cell groups could migrate as a group or they could also arise from divisions of an originally single cell. Furthermore, we observe MV positive cells anterior in the mesoderm layer at the level where the PS is FOXA2 positive. A description of these observations was not included in the main text.

As a minor comment, and although the paper is very well constructed and written, I would suggest to perhaps try and be a bit more concise and more precisely separate the specific conclusions from the different approaches as it feels slightly repetitive at times.

We thank the reviewer for this comment. We adjusted the text to be more concise in our writing and removed sections that appeared redundant.

Reviewer 2
Probst et al use a combination of imaging of reporter lines, immunohistochemistry, and single cell transcriptomics to argue for that Eomes positive progenitors in the epiblast give rise to independent lineage specified progenitors for endoderm and anterior mesoderm in a spatial and temporally distinct manner. They employ a set of reporters (Eomes-membrane Tomato/ nuclear GFP and Mesp1-membrane Venus), their own limited single transcriptomics, FoxA2 antibody staining, a CreERT-Eomes allele and re-analysis of a larger transcriptomic dataset from Bertie Göttgens lab. For the transcriptomics analysis they employ some interesting new tools including RaceID and FateID, in addition to generating a useful new reporter mouse line, the Mesp1-membrane Venus.

The fundamental observation contained in this paper, that AM and DE arise independently is a nice confirmation of the clonal analysis from Val Wilson's and previous fate maps. As they have not considered the axial mesoderm and endoderm, it is hard to make a strong conclusion about the absence of a common precursor of mesoderm and endoderm outside of the epiblast, so their conclusions are relatively limited. If this were the only issue, perhaps one could consider publication in Development. However, there are a number of additional issues with the manuscript that make it difficult to evaluate their conclusions. Thus, their conclusions about Eomes uniformity, FOXA2 expression, Mesp1/FOXA2 co-expression are based on individual images without any quantification. In addition, the single cell transcriptomics is relatively superficial. The manuscript contains lots of tSNEs, but little in depth analysis and incomplete cluster annotation. The authors wish to make a

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conclusion about progenitor specification, using sophisticated reporters, but with a complete absence of live imaging. Finally, there are number of confusing and sweeping statements that are lacking in significant substantiation. For all these reasons, this manuscript would seem too preliminary for publication or consideration in a reasonable period of revision.

As this section contains several critiques we answer them separately:

– *We failed to find the published papers by Val Wilson that the reviewer is referring to. To our knowledge Wilsons's studies investigate lineage separations at later timepoints. Thus, we could not compare with and refer to suggested publication(s).*

– *Reviewer 2 mentions correctly that we did not investigate the separation of axial mesoderm (AxM) and DE, which both express Foxa2. We can therefore not make any conclusions about whether there exists a Foxa2 positive common progenitor for these two lineages. We now included a sentence to the discussion to clarify this distinction (lines 470 - 476). In our study we rather focus on the earliest population of AM as clearly specified throughout the text. As both DE and AxM are epithelial tissues generated in close proximity in the APS under the influence of high levels of Nodal signaling it would be interesting to investigate in further studies if they share a progenitor.*

– *To improve the documentation of uniform Eomes expression in the epiblast and to identify the position of Mesp1/Foxa2 double positive cells we have included additional experimental data as described in point 3, point 16 and reviewer 3 major point 1.*

– *All IF anaylses presented were performed at least in triplicates and were not based on single images. Mesp1/Foxa2 double positive cells were quantified using the scRNA-seq data, that we consider more suitable for quantification of cell types than imaging data.*

– *We adjusted the cluster annotation of the scRNA-seq data for clarification as also described in points 7 and 8 (below). While t-SNE and UMAP plots indeed may seem repetitive, we believe that this is currently the best way of representation of scRNA analyses, including fate bias analysis (Fig. 6). Additionally, we have performed further analysis of Mesp1/Foxa2 double positive cells, as both Reviewer 2 and 3 have appropriately pointed to the fact that these cells could also represent bipotent progenitors. This issue we discuss in point 16 and also in our response to the comments from Reviewer 3.*

– *We carefully checked our manuscript to be more concise, removed redundant sections as also pointed out by Reviewer 1, and aimed for not stating any unsubstantiated conclusions.*

1. The writing and abbreviations are confusing. Some abbreviations are not defined.

We carefully checked the text for all abbreviations used and made sure that they are now defined in the right place. Additionally, we went over the whole and amended potentially confusing sections.

2. In Fig 1, they localize mesoderm and endoderm progenitors, but do not explain how these are defined.

In the IF images mesoderm and DE cell layers are defined by their location. At E6.5 and E7.25 the mesoderm layer contains both mesoderm and DE progenitors (Viotti et al, 2014). We have changed the labeling ME to mesoderm (Mes). By E7.5 intercalation of DE into the VE layer is mostly completed (Kwon et al., 2008), therefore we assigned the identity of DE to the outer, endoderm layer at this stage. We have changed the figure legend of Fig. 1 accordingly. The identities of the scRNA-seq clusters are assigned by their gene expression (Table S1 and S2).

3. On line 136 the authors state that all cells leaving the primitive streak are Eomes positive. However, in the referenced figures, they show no quantification and immunohistochemistry is based on single example sections only. Better quantification is required for this conclusion.

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To better demonstrate that all cells leaving the PS during the first day of gastrulation are EOMES positive we have sectioned and stained whole embryos at E6.5/E6.75 (n=6) and E7.25 (n=5) for EOMES protein. Series of embryonic sections are provided as Supplemental movies 1 and 2. These show that all cells leaving the PS are positive for EOMES. In the distal regions of the posterior epiblast/PS we rarely identified any cells that are negative or show very low expression of EOMES. However, all cells that have delaminated from the epiblast are positive for EOMES. The movies have been added to the supplementary material (Supplemental movies 1 and 2).

4. On line 140, they state there are some reporter negative cells in the endoderm, and state these are “most likely,” VE cells that lose reporter expression. However, no demonstration that these are VE cells has been presented. They also maintain elsewhere that Eomes is expressed throughout the VE, does this mean it is down regulated in the VE more rapidly than the DE?

Many thanks for noting that the expression dynamics of Eomes in the VE were described confusingly. It has been published that EOMES is present in the embryonic VE at E5.5 (the extraembryonic VE is at this point negative for EOMES) (Nowotschin et al., 2013). Our analysis shows EOMES negative cells in the VE starting at E6.5 (see Movie S1). These EOMES-negative cells are VE, as no DE cells have egressed into the outer layer at this point. This is now explained in the main text (lines 140-143).

5. The color scheme that the authors have chosen for the immunohistochemistry is very difficult to interpret.

We were unable to identify the Figure that Reviewer 2 was referring to. Thus, we have not changed the colors in the IF images, but would certainly do so if this criticism could be specified.

6. In supplementary Fig 1A and B, there are clusters that appear composed of one or two cells (or even no cells).

There are clusters with few (even up to a single cell) as mentioned in the main text (line 162). This is due to the RaceID algorithm which is designed to find rare cell populations which consist of only few cells. There are no clusters without any cell.

7. In Fig 1I and L they did not assign the cluster identity for all the clusters. In L, there are clear clusters that were not assigned and it is not clear why these are merely defined as “other.”

In Figure 1I and L we only specified the identities of clusters that are of interest to this study. For completeness we now have assigned the identities of all clusters in Figures S1A and B.

8. There number of clusters defined in the supplemental data are different from those defined in the main figures.

In main Figure1 we assigned identities to clusters (in some cases more than one cluster belong to one identity). In Figure S1 all the clusters identified are shown. As mentioned in point 7, we have annotated all clusters in Figures S1 A, B.

9. On line 175, they say there is no sub-cluster within the E6.75 epiblast, but they show sub-clustering of epiblast in S1.

It is correct that RaceID identified two clusters with epiblast identity. However, analysis of differentially expressed genes between these two clusters did not indicate any clearly distinct progenitor populations. The two clusters were similar to each other, only few genes are differentially expressed between these two clusters. These data are available in Table S1. We have referenced Table S1 in Figure legend S1 to make it clear how the identities were assigned. In the specific text section on line 175 (now line 167) we didn’t refer to actual identified clusters, but rather the separation on the t-SNE maps. Therefore, we have slightly rephrased and rearranged the section.

10. They have introduced a bias by hand picking cells at E6.5, but using FACS for E7.5. Why introduce this bias? Why not included FACS sorted cells at E6.5 to control for this bias? We imagine that the different experiment approaches here would introduce technical batch variation. Is this why they have not analyzed their data together? Combining these stages would seem important, as

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progenitors are specified across a window of developmental time.

We performed handpicking at E6.75 because embryos at this early stage do not have sufficient numbers of cells for performing a FACS based approach. Indeed, batch effects are frequently observed in this type of approaches. However, the most significant batch effects was observed within the E6.75 timepoint between cells picked at different experimental dates and we aimed for correcting all batch effects before and while analyzing the data.
We also combined our datasets from different developmental stages. However, we rather focused this analysis on the larger published scRNA-seq dataset.

11. From line 191 they claim embryonic Eomes positive cells at E6.75 are quite "similar," to each other. However, there is no correlation data to support this. Nor is Eomes included in the heat maps they generated. In 1K, Eomes expression appears quite heterogeneous.

We agree that the phrasing was unclear and rephrased the summary of Figure 1. Accordingly, embryonic cells at E6.75 (as compared to extraembryonic cells) cluster closely together, indicating that they are molecularly similar (line 181 - 184).

12. On line 208 they claim they identify the earliest Eomes dependent mesoderm at E6.5, but its hard to fathom how they can make this claim in the absence of either live imaging or extensive quantification. A similar statement is made on line 212.

We have removed the word 'earliest' from the sentence in line 208. On line 212/213 (now lines 201 – 202) we state that most mesoderm cells observed in E6.5 embryos are Mesp1^{mV} positive, which is clearly visible in Figure 2E. Again, this statement is not based on a single image, but we have looked at several embryos, in this case n = 9.

13. On line 220, they also make statements about numbers of Mesp1 negative cells, but its hard to understand how they can make this conclusion based on the data in Fig 2I and L.

This is a valid comment, we have changed the sentence and we are now only stating that Mesp1^{mV} negative cells are also found mixed within the positive cells (line 209 – 210).

14. At E6.5, line 246, they claim that FOXA2 expressing cells are DE progenitors. How do they know? FOXA2 marks a spectrum of lineages including axial mesoderm and floor plate.

Indeed FOXA2 positive cells could also represent AxM progenitors. We have included a section in the discussion addressing the fact that we did not investigate the AxM population, which is part of the FOXA2 positive population. However, the analysis of Burtscher et al. showed that FOXA2 and T domains are separated at E6.5, indicating that the FOXA2 positive cells at E6.5 are indeed DE progenitors, because AxM progenitors would be expected to be T positive (Burtscher et al., 2009). *Only at timepoints later than E6.5 the authors describe the presence of T positive (mesoderm), FOXA2 (DE) positive and T/FOXA2 double positive cells (AxM).*

15. In Fig 3B they point to a region of the embryo and say it is DE. Why?

-This region is the APS, where DE (and AxM) arises. We see increased density of FOXA2 positive cells here which we assume are these progenitors as stated in the figure legend (line 830 – 832).

16. Around line 256, they claim that there are only "rare," Mesp1/FOXA2 double positive cells. However, in the single transcriptomics in Fig S4, the double positive population appears quite extensive. Does this represent a discrepancy? They claim these could be progenitors of the cardiac ventricles, but there is no data to support this. Furthermore, on line 260, they dismiss their identity as a bipotent progenitor population without providing any data and based on their position at the junction of two domains of expression.

We don't believe that there is a discrepancy between the imaging and the scRNA-seq data. The scRNA-seq data contains cells from several embryos. For example, the percentage of double positive cells within all Mesp1 and Foxa2 positive cells at E6.75 is 3.4%. The quantification of numbers of

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Mesp1/Foxa2 double positive cells is presented in Figure S3E and these cells seem generally "rare". We now analyzed these cells more extensively and gave the possibility that these double positive cells could be bipotential progenitors more thought, as this was additionally requested by Reviewer 3. We have addressed this request by additionally staining whole embryos for FOXA2 and Mesp1^{mV} to show where they are localized (Supplementary Movies S3 and S4). To transcriptionally characterize these cells we have performed additional analysis of scRNA-seq data (Supplementary Fig. S4C, D). This is also discussed in the response to Reviewer 3, major point 1.
*We mention that these could be progenitors to the cardiac lineages because these data have been published elsewhere (*Bardot et al. 2017; Ivanovitchet al. 2020).

17. In Fig 4, they claim to harvest embryos from E6.25-7.5, culture for 90 minutes with 4OHT and then culture for 24 hours. How did they collect the samples for E6.5? Where are the embryos cultured for 24 hours from E7.5? The methodology is very unclear.

As described in the material and methods section the embryos were dissected from the uterus at the indicated timepoints. To demonstrate how these embryos looked at the start of the culture and after 90 min + 24 hrs, we are providing images from the start and end of the embryos for some examples, including some fluorescent images taken at the end (please see images below). In the main text we always refer to the age of the embryo at the timepoint of labelling, e.g. at the start of culture. We hope this explains the approach more clearly.

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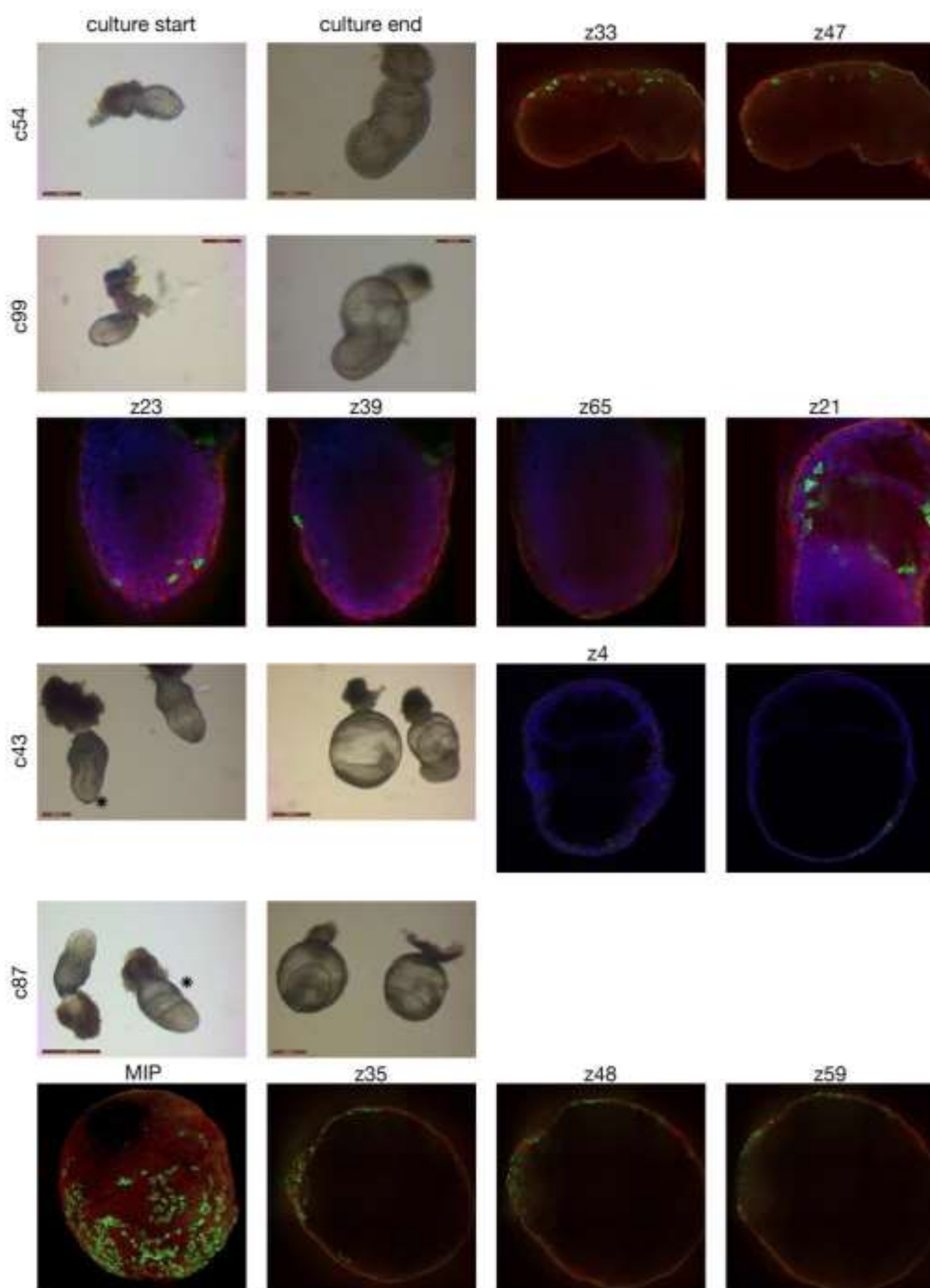

***Figure 'Examples of cultured embryos'**: some example embryos of the genetic lineage tracing are shown. Images of the embryos at the start of culture (after tamoxifen treatment) and after 24 hrs of culture. Additionally, examples of optical sections (indicated as z for section plane) are shown.*

18. As they have clear defined beginning and end points for their analysis, it is not clear why they have not generated pseudotime on the transcriptomic data, so that their work could be benchmarked against the analysis coming from other groups.
We feel that pseudotemporal analysis, which might additionally include a bias by the analysis itself, was not required for our analysis exactly because of the reason that we have real temporal data. This is

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especially the case for the large published dataset.

19. On line 289 they say that upto 5% of the cells could be double positive for Mesp1/FOXA2. This would seem a reasonable percentage of cells for a progenitor population.

This pint was already addressed above, please see point 16 and major point 1 of Reviewer 3.

20. While Mesp1 and FOXA2 are good markers, one wonders why they can't exploit all of this transcriptomic data to find additional markers that could be used to strengthen any conclusions.

We had indeed considered additional markers for the presented study but decided on the use of Mesp1 and Foxa2, since these two are among the earliest robustly expressed markers and were therefore best suited for our analysis. Of course, scRNA-seq data allow to search for additional early lineage markers and our analyses also identified potential candidates (Fig. S5A). However, we did not investigate these further as this was not in the focus of this study.

21. The application, FateID, is not explained. It would appear just a variation on pseudotime. The target population in Fig 5I, J appears to have a significant set of overlapping cells. If this is not the case, then these target populations need to be better represented. They also appear to pool cells from E6.5/E6.75, but not other stages. Is this a good idea?

We have included a brief explanation of FateID in the text (lines 390 - 395). For further details about the method we refer to the publication explaining it (Herman et 2018).
The target populations in Fig. 5I are actually not overlapping since it did not contain Mesp1/Foxa2 double positive cells. These are indeed Mesp1 or Foxa2 positive cells clustering closely together.
We pooled the cells from E6.5 and E6.75 to increase the numbers of cells at these early stages. This should be a feasible approach because these two stages look very similar by the IF analysis. For later timepoints it was not required to combine them, because scRNA-seq data of more cells were available at different stages.

22. What is the meaning of the idea that Eomes single positive cells are biased in a particular direction? Is this based just on gene expression similarities? They dismiss the notion that these trajectories could represent distinct progenitors, based on the differential gene expression not revealing any known markers of either lineage, but perhaps there are new markers in their dataset.

The possibility of distinct progenitors for early mesoderm and DE is discussed (lines 510 – 513). However, scRNA-seq analysis of Eomes expressing, lineage-marker negative cells did not show clear subclusters (Fig. 1K) suggesting that Eomes-expressing progenitors are rather uniform.
FateID identifies subtle transcriptome differences that could indicate a lineage bias. The algorithm does not distinguish between know and new markers as it takes all expressed genes into account. This iterative method starts with the target population, in our cases Mesp1 or Foxa2 positive cells, and moves backwards by calculating similarities of the neighboring cells to the target clusters. The most similar cells are then included in the next iteration, when cells neighboring these cells are calculated. This is done until all cells have been analyzed. FateID gives probability scores between 0 and 1 for all progenitor cells (which are the Eomes single positive cells) towards each target population. A probability score of 0.5 would mean there is the same probability to reach either fate, resulting in no fate bias.
To calculate the differentially expressed genes of cells fated towards one target population, we took a low cut-off of 0.6 probability score. This analysis resulted in only few genes that are changed between these two populations (Fig. S5E). These genes could indeed include novel markers for the epiblast progenitor population as suggested by Reviewer 2.
However, the fact that most cells have a similar probability for both lineages suggests that these are not yet biased and most likely present a uniform cell population.

23. There is a good FOXA2-Venus reporter, why have the authors not used this. It is a fusion protein and therefore largely nuclear.

We are aware of this Foxa2-Venus reporter. The staining for FOXA2 protein is working very well and as such no reporter strain was required. Additionally, the reporter consists of a Venus fluorescent protein, same as the Mesp1-reporter, which makes it less useful for our purpose.

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24. In the discussion they say that almost all cells leaving the primitive streak are Eomes positive. In the results, it is all cells. It would seem difficult to conclude all cells express Eomes based on the data in S3. What then are the Eomes negative cells? As mentioned in the earlier comment, it is also difficult to assess how the cluster were assigned and how Eomes is expressed across each of these clusters.

As mentioned above (point 3) we performed additional experiments and present the data that all cells leaving the PS between E6.5 and E7.25 are Eomes positive. The wording in the discussion was changed accordingly.
Eomes negative cells in Fig. S3 represent the cells that have migrated away from the PS and downregulate Eomes expression eventually. As shown previously Eomes RNA levels are very dynamically regulated and by E7.5 cells of the PS start to downregulate Eomes mRNA (Ciruna et al. 1999). Obviously, there is a striking discrepancy between protein and RNA expression.
As for our data, the clusters identities were assigned according to genes expressed in each specific cluster. We show the distribution of Eomes expression across the cell identities (Fig. S1C, D), but did not repeat this data for the published dataset.

25. In multiple figures they discuss coexpression and correlation, but fail to produce a regression line or correlation score. See for example, Fig 3N.

In Fig. 3N we did not analyze the correlation of gene expression (levels), but how genes are co-expressed. Nevertheless, we have calculated the correlation scores for the plots in Fig. 3N.
Correlation between Mesp1 and Foxa2: -0.0782863 Correlation between Foxa2 and Eomes: 0.2262012
Correlation between Mesp1 and Eomes: 0.3991671

The correlation between Mesp1 and Foxa2 is very low as expected, as the two genes are mostly expressed in separate cells.
The correlation between Eomes and Foxa2 or Mesp1 is somewhat intermediate, which is also expected, as there are co-expressing cells but also Eomes single positive cells that have not yet started to express lineage markers. We have added these correlation scores to the figure legend.

Reviewer 3

The study by Probst et al addressed the molecular and cellular basis of one of the earliest cell fate decisions in the mammalian embryos, the segregation of the endoderm (DE) and mesoderm (AM) lineages during gastrulation. This is an important issue with several unresolved questions that the authors address. Using high resolution imaging, genetic lineage tracing and single cell transcriptomics they examine the emergence of the AM and DE from Eomes- expressing cells in the primitive streak. In addition to rigorously confirming previous observations on the temporal-spatial dynamics of DE vs AM. Specifically they examine whether or not these two lineage emerge from a common “mesendoderm progenitor” which has been postulated mostly from in vitro differentiation of pluripotent stem cells. In general the quality of the data is excellent, particularly the embryo staining and lineage tracing. However I have some reservations about the authors conclusions and with their assumption that the common progenitor would have to be a large population of cells that co-express Foxa2 and Mesp1. I strongly suggest that the authors include a more balanced interpretation of the data and provide additional analysis that would reflect the alternative hypothesis. If the authors can address my concerns, then I feel that this will be a valuable contribution to the field.

We thank reviewer 3 for this constructive criticism. We have further analyzed and discussed the Mesp1/Foxa2 double positive cells as potential bipotent progenitors as described below and aimed for a more balanced discussion as suggested.

Major comments:

1. The main conclusion that they don’t find any evidence of a Foxa2/Mesp1 co- expressing cell population is simply not true. Their analysis of the Pijuan- Sala data (which has sufficient cells) show that 2-5% of the cells are Foxa2/Mesp1 double positive. The authors explain this away with the

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statement, "Their location only at the border of Mesp1 and Foxa2 positive areas suggests that these are specialized cells rather than bipotential progenitors." While this is possible, the data also support an alternative interpretation, where the bipotential progenitor is very transient with the respective DE and AM GRNs mutually repressing each other rapidly. This would be equally interesting. I suggest presenting the data and both interpretations.

We much appreciate this comment by Reviewer 3. The relatively low numbers of Mesp1/Foxa2 double positive cells obviously don't exclude them as bipotential progenitors. To address this interesting question, we have further investigated the double positive cells by more thoroughly locating the Mesp1/Foxa2 double positive cells within the embryo (reanalysis of stained embryos and additional IF staining of sections from whole embryos, movies S3 and S4). We have also analyzed the transcriptional state of these cells in the large published scRNA-seq data set (Figure S4C and D). Finally, we provide some additional lineage tracing data performed with a Mesp1Cre line in conjunction with a LacZ-reporter (Figure S4 G).
Reanalysis of the presence of these cells shows that these are localized around the intermediate level of the PS. Most of these cells reside in the mesoderm layer, and some rare cells can be found in the PS itself. A description of these data was included in the main text (lines 240 ff). While the localization of Mesp1/Foxa2 double positive cells alone does not necessarily exclude that these are bipotential it is highly unlikely that these represent progenitors for the majority of early generated mesoderm and DE. While we find Mesp1/Foxa2 double positive cells at intermediate PS levels we see in proximal regions only Mesp1^{mV} positive cells in the PS and in the delaminated mesoderm cells, and in distal PS regions and in delaminated cells only Foxa2 positivity.
These mesoderm and DE progenitors very likely do not originate from the double positive population that doesn't seem like a transient progenitor population for the majority of AM or DE cells. It could however be a progenitor population for a specific subpopulation. We now included a more extensive section in the discussion.
The scRNA-seq analysis of Mesp1/Foxa2 double positive cells indicates that these cells express markers of both AM and DE lineages, which could fit with a role as bipotential progenitors. The clustering of these cells and visualization of expressed markers for AM, DE and epiblast indicated that these cells are not uniform. Rather, they represent the continuum of a specific lineage (i.e. a subpopulation of the heart). This information is described in the main text (lines 374 - 385) and included in the new Fig. S4. We also include images (Fig. S4G) of embryonic lineage tracing using the Mesp1Cre mouse line for fate- labelling (Saga et al. 1999) *and references to published data, showing that fate-labelling by Mesp1.Cre does not result in labeling of the DE lineage (Fig. S4G).*

2. In Fig. 5 the authors should include a separate UMAP plot showing Foxa2/Mesp1 double positive cells. In Fig 5G-J there is a population of double positive cells at the DE-AM branch point that appears to be biased to both Mesp1 and Foxa2 fates. Indeed the authors say .." The cells closer to the branching point mostly did not display a clear fate bias showing an intermediate probability for both lineages". This is precisely the predicted behavior of the common progenitor, so I don't understand why the authors dismiss this interpretation.

We now have included a UMAP plot showing the location of the Foxa2/Mesp1 double positive cells (Fig. 5C). These cells are scattered across the different populations, but are enriched in the distal PS. The target cells (in red) in now Fig. 6E-H that are localized around the branch point are actually either Mesp1 or Foxa2 expressing, and the double positive cells were excluded from this analysis. The cells used to calculate the potential fate bias to either lineage are negative for both Mesp1 and Foxa2, but represent the Eomes single positive cells. These cells at the branch point don't show a clear fate bias and we think these might be the common bipotential progenitors for AM and DE. These cells do not co-express lineage markers, but are immediately assigned to the one or the other fate. So, we don't claim that there is no mesendoderm progenitor, but want to propose that this progenitor is the Eomes single positive cell of the posterior epiblast. Alternatively, there could be also spatially separated progenitor populations within the Eomes single positive population as described above (Reviewer 2, point 22). This possibility is also discussed in the text (lines 507-513).

3. The assumption that a common biopotential progenitor would form a separate cluster is a bit of a stretch. It might, but it might also simply fall in a continuum of cell states between PS DE and AM, which is what my interpretation of the data show. In this case the Eomes-expressing PS cells are the

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common progenitor and as cells ingress through the streak they very transiently express markers of both lineages as they segregate.

We agree with Reviewer 3 that the bipotential progenitors could be included in the different clusters of Eomes positive cells. Also, we concur with Reviewer 3 that the Eomes positive cells of the posterior epiblast most likely represent the bipotential progenitors. We modified the text to bring across this interpretation more clearly. However, while we aimed for a more balanced discussion we still favour out initial interpretation of the data so that most cells ingressing through the early PS will not go through a state of expressing both, AM and DE, lineage markers (as mesendoderm progenitor).

Minor comments:

4. In Fig.4 the authors should include the EmM scoring data in the Fig 4E histogram. This would help directly comparing DE vs Mes contribution over time.

We have included a bar graph as Fig. 4F showing that all embryos have mesoderm labeling. The contribution of embryos showing only extraembryonic mesoderm or only embryonic mesoderm labeling is indicated.

5. In the discussion a brief consideration of the support for a common progenitor from PSC differentiation literature would be helpful. Perhaps an artifact of in vitro, or perhaps the cultures allow one to isolate the transient nature of cells in the primitive streak.

We have included a section in the discussion about the Mesp1/Foxa2 double positive cells and ESC experiments suggesting the existence of a co-expressing state (lines 478 – 498).

6. The authors need to more clearly acknowledge the limitations of their own scRNA-seq data set. While manual isolation of the cells is an impressive example of embryology, the fact that they have less than 20 DE and AM cells makes it really impossible to draw robust conclusions considering the transcript dropout. Of course they mostly use the Pijuan-Sala data, which is appropriate, but they should acknowledge this more directly.

We have added a sentence mentioning that we used the published data set due to the limited numbers of cells in our data set on (line 258).

7. Although I am versed in both the embryology and scRNA-seq applications, I cannot assess the actually computational work and recommend a reviewer with expertise in that area. In general the robustness of the scRNA-seq analysis would be enhanced if alternative methods of trajectory inference, such as Monocle or URD showed similar results.

We have identified similar clusters and gene expression profiles for both scRNA-seq data sets we analyzed, and also received comparable clustering from the primary data as previously published. This suggests that our analysis is robust and reproducible. It is noteworthy, that two of the co-authors, namely Sagar and Dominic Grün are specialist in the analysis of single cell data sets and at the forefront of method developments.

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Second decision letter

MS ID#: DEVELOP/2020/193789

MS TITLE: Spatiotemporal sequence of mesoderm and endoderm lineage segregation during mouse gastrulation

AUTHORS: Simone Probst, Sagar, Jelena Tosic, Carsten Schwan, Dominic Gruen, and Sebastian J. Arnold

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 positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Referee 2 has several points, most of which are requests for clarification or changes to the presentation of data. The additional piece of analysis requested is the quantification of Foxa2, Eomes and Mesp1 protein/reporter levels in embryos. I agree with the referee that this would strengthen the conclusions from the sc-RNA-seq and I would encourage you to include these data.

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.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. 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.

Reviewer 1

Advance summary and potential significance to field

The paper adequately adresses the question of separation of the mesoderm and endoderm lineages at the primitive streak. It adds useful data to suggest that the lineages are separated from an eomesodermin positive common progenitor in the PS, essentially without a biprogenitor state (which could anyhow be present as a rare event).

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Comments for the author

The authors have adequately addressed my comments both through additional experiments and analysis and through more careful wording of their findings.

A last revision effort might be done, in particular in the added sentences, in using simpler sentences or cutting the longer ones in individual ideas, to avoid any ambiguity in their significance (in all transparency, I am not a native speaker, so perhaps this is my own limitation).

Reviewer 2

Advance summary and potential significance to field

The fundamental issue this paper seeks to resolve, is one that is generally assumed to be true by developmental biologists, but not in the stem cell field. This is the reason for our original reference to Val Wilson's work, where in Tzouanacou et al 2009, their clonal analysis points to a relatively low number of multi lineage clones containing mesoderm/endoderm, but not ectoderm. While they do not rule out the existence of rare mesendodermal precursors, they argue against an "obligate mesendoderm-specific progenitor." Yet, despite the paucity of developmental evidence for "mesendoderm" (a common progenitor of the mesoderm and endoderm) in developmental studies, it is commonly accepted in the embryonic stem cell (ESC) field. For this reason, this demonstration of the absence of such a common progenitor would be appropriate for publication in Development. As we said in our original review, the main issue with the original manuscript was the preliminary nature of some of the initial analysis. In the revised manuscript there is much improvement, although there remains one major issue that should be addressed prior to publication. Currently, the visualization of progenitor populations depends on a set of images with no quantification and transcriptomic data that removes spatial information. Given that the images are available, why not provide quantification? In our original review we requested quantification of the imaging, and we still believe would represent substantial improvement to the manuscript and would like to see this included prior to publication. Quantification of the double and single positive (Foxa2, Eomes and Mesp1 populations) in vivo alongside the single cell transcriptomics would not only strengthen the claims in the manuscript, but also be of general interest to the readership of Development.

Comments for the author

Most of our original comments were addressed, we list a few outstanding issues below.

Specific Comments:

1. The quantification of Foxa2, Eomes and Mesp1 protein/reporter levels in embryos will strengthen the conclusion produced by their sc-RNA-seq. As they have such well performing antibodies, reporters and an abundance of sections, some level of quantification should be possible. In their own response to our comments, they discuss the dynamics of Eomes expression and the discrepancies between protein and RNA expression. The suggested quantification of their embryo staining would provide a better context with which to view the quantitative cell transcriptomic classifications of distinct progenitors.

2. Although we understand the authors response to point 10 in our review and do not expect them to collect more data at this point, we wish to point out that it is very feasible to sort cell from E6.5 embryos, when carrier cells are used.

3. The discussion of the transient mesendoderm progenitor from line 445 really highlights how confounding experiments in ESCs have been. Perhaps the concept of "mesendoderm," could also be raised in the introduction. Either the paragraph starting at line 63 or the one beginning on line 78.

4. In response to the authors query about comment on the color scheme. We should have been more specific. Apologies. Our point was that DAPI as dark blue and magenta (e.g. Fig. 1G, H) or

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blue and green (better, but could be clearer, e.g. Fig. 2, D, G, and H) are difficult to distinguish on a black background, particularly in print. A suggestion would be use white for the DAPI.

5. In the legend to Figure 4, could the authors make clear that the times in the tables and plots represent time of tamoxifen treatment.

6. After close inspection of the new Fig 6G and H (formerly 5I, J) we agree with the authors that there is little overlap. Perhaps the panel could be enlarged slightly or an inset for the middle region at higher magnification included, to emphasize this.

7. As we suggest in our point 22, the notion that these cells already exhibit fate bias is interesting, but why are they different? The say that only a few genes vary between the populations in Fig. S5D, E. However, perhaps these genes could be included as a supplemental table or heat map. Alternatively, they could add a sentence or two along the lines of what was included in their response to our comments.

Reviewer 3

Advance summary and potential significance to field

I am satisfied by the revisions which have improved the data analysis and provide a more balanced interpretation. This is an valuable contribution that addresses an important question in the field of early vertebrate development, namely the origin of the transient bipotential mesendoderm progenitor that gives rise to endoderm and mesoderm lineages.

Comments for the author

I appreciate the effort that the authors made to address my concerns.

Second revision

Author response to reviewers' comments

Point-by-point response to Reviewers' comments

Reviewer 1 Advance summary and potential significance to field
The paper adequately adresses the question of separation of the mesoderm and endoderm lineages at the primitive streak. It adds useful data to suggest that the lineages are separated from an eomesodermin positive common progenitor in the PS, essentially without a biprogenitor state (which could anyhow be present as a rare event).

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We went through the text again and adjusted sentences where necessary.

Reviewer 2 Advance summary and potential significance to field
The fundamental issue this paper seeks to resolve, is one that is generally assumed to be true by developmental biologists, but not in the stem cell field. This is the reason for our original reference to Val Wilson's work, where in Tzouanacou et al 2009, their clonal analysis points to a relatively low number of multi lineage clones containing mesoderm/endoderm, but not ectoderm. While they

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do not rule out the existence of rare mesendodermal precursors, they argue against an “obligate mesendoderm-specific progenitor.” Yet, despite the paucity of developmental evidence for “mesendoderm” (a common progenitor of the mesoderm and endoderm) in developmental studies, it is commonly accepted in the embryonic stem cell (ESC) field. For this reason, this demonstration of the absence of such a common progenitor would be appropriate for publication in Development. As we said in our original review, the main issue with the original manuscript was the preliminary nature of some of the initial analysis. In the revised manuscript there is much improvement, although there remains one major issue that should be addressed prior to publication. Currently, the visualization of progenitor populations depends on a set of images with no quantification and transcriptomic data that removes spatial information. Given that the images are available, why not provide quantification? In our original review we requested quantification of the imaging, and we still believe would represent substantial improvement to the manuscript and would like to see this included prior to publication. Quantification of the double and single positive (Foxa2, Eomes and Mesp1 populations) in vivo alongside the single cell transcriptomics would not only strengthen the claims in the manuscript, but also be of general interest to the readership of Development.

Reviewer 2 Comments for the author
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Specific Comments:

1. The quantification of Foxa2, Eomes and Mesp1 protein/reporter levels in embryos will strengthen the conclusion produced by their sc-RNA-seq. As they have such well performing antibodies, reporters and an abundance of sections, some level of quantification should be possible. In their own response to our comments, they discuss the dynamics of Eomes expression and the discrepancies between protein and RNA expression. The suggested quantification of their embryo staining would provide a better context with which to view the quantitative cell transcriptomic classifications of distinct progenitors.

We have now included a quantification of cell numbers of Mesp1mV, FOXA2, and Mesp1mv/FOXA2 double positive cells on whole embryo sections (Fig. S3E, main text lines 284-286, material and methods). This quantification fully confirmed the analysis done by scRNA-seq

2. Although we understand the authors response to point 10 in our review and do not expect them to collect more data at this point, we wish to point out that it is very feasible to sort cell from E6.5 embryos, when carrier cells are used.

We thank the reviewer for this suggestion. In addition, the experimental approach developed by 10XGenomics could now be applied. However, this methodology was not available to us at the time of the experiments of this study.

3. The discussion of the transient mesendoderm progenitor from line 445 really highlights how confounding experiments in ESCs have been. Perhaps the concept of “mesendoderm,” could also be raised in the introduction. Either the paragraph starting at line 63 or the one beginning on line 78.

We appreciate this suggestion and now included a sentence leading to the concept of the mesendoderm in the introduction (now lines 66-68).

4. In response to the authors query about comment on the color scheme. We should have been more specific. Apologies. Our point was that DAPI as dark blue and magenta (e.g. Fig. 1G, H) or blue and green (better, but could be clearer, e.g. Fig. 2, D, G, and H) are difficult to distinguish on a black background, particularly in print. A suggestion would be use white for the DAPI.

We agree with the reviewer that the DAPI staining is sometimes not well visible. We now tested if changing the color of the DAPI staining to white would enhance visibility. However, with this type of representation we loose contrast to the green channel. Therefore, we suggest to keep the blue color for the DAPI channel, which was increased in brightness in images where it appeared too dim.

5. In the legend to Figure 4, could the authors make clear that the times in the tables and plots represent time of tamoxifen treatment.

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We have added a sentence to the figure legend (lines 881-882) clarifying this issue.

6. After close inspection of the new Fig 6G and H (formerly 5I, J) we agree with the authors that there is little overlap. Perhaps the panel could be enlarged slightly or an inset for the middle region at higher magnification included, to emphasize this.

We considered introducing a zoomed view of the UMAP representation. However, this removes graphical orientation and might be misleading. We checked that the quality of the figure is sufficient to electronically zoom-in on the region in the current Figure, which might be the preferred way of closer inspection.

7. As we suggest in our point 22, the notion that these cells already exhibit fate bias is interesting, but why are they different? The say that only a few genes vary between the populations in Fig. S5D, E. However, perhaps these genes could be included as a supplemental table or heat map. Alternatively, they could add a sentence or two along the lines of what was included in their response to our comments.

We state that only very few genes are changed between the cells from Fig. 6C, D (not Fig. S5D, E) that have fate probability of 0.6 or more towards either Mesp1 or Foxa2 positive cells. In Fig. S5A the genes changed more than 2-fold are indicated and a full list is provided in Table S7 as also mentioned in the main text lines 412-415. In fact, we interpret this result in such a way that these cells are not very different at early timepoints and therefore not truly biased.
At the later timepoint, we find a clearer bias and this is also visible in the genes changed (Fig. S5A and TableS7).
We apologize that in our response to point 22, we referenced the wrong panel, Fig. S5E instead of Fig. S5A, this might have created some confusion.

Reviewer 3 Advance summary and potential significance to field
I am satisfied by the revisions which have improved the data analysis and provide a more balanced interpretation. This is an valuable contribution that addresses an important question in the field of early vertebrate development, namely the origin of the transient bipotential mesendoderm progenitor that gives rise to endoderm and mesoderm lineages.

Reviewer 3 Comments for the author
I appreciate the effort that the authors made to address my concerns.

Third decision letter

MS ID#: DEVELOP/2020/193789

MS TITLE: Spatiotemporal sequence of mesoderm and endoderm lineage segregation during mouse gastrulation

AUTHORS: Simone Probst, Sagar, Jelena Tosic, Carsten Schwan, Dominic Gruen, and Sebastian J. Arnold
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.

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