# Single-cell RNAseq analysis of testicular germ and somatic cell development during the perinatal period

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**Original submission**

First decision letter

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MS TITLE: Pro-spermatogonia development and spermatogonial stem cell genesis defined at single-cell resolution

AUTHORS: Kun Tan, Hye-Won Song, and Miles Wilkinson

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

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. Specifically, the referees identify three main areas which they believe need to be addressed:
- A more thorough comparison of your data with recently published similar single cell transcriptome analysis (including Law et al 2019).
- Provide more analysis of cells in the somatic compartment.
- Provide functional analyses that test predictions of arising from the analysis of the transcriptome data.
I realise this represents a substantial revision to your study and I would be happy to discuss a revision plan with you, if that would be helpful.

If you are able to revise the manuscript along the lines suggested, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing

how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

*Advance summary and potential significance to field*

The authors used scRNAseq analysis to characterize gene expression in the major cell types in the testes during the perinatal period. They examined the transcription of SSC precursor cells—ProSG—as they differentiate, migrate, undergo proliferative expansion, and convert into SSCs. They identified a new putative ProSG subset they suggest is a migratory cell that moves from the center of the tubule to its basement membrane, where it begins to proliferate and presumably becomes SSCs. They also identified a primitive SSC subset and suggest that it is the direct descendent of ProSG. Marker proteins for the ProSG stage and first SSCs were identified. We also defined transcription differences and gene markers for testicular somatic cell types. They propose ligand and receptor genes encoding signaling pathways expressed in germ and somatic cell subsets.

*Comments for the author*

Line 51 “subtle”
Fig 2 and specific markers for T1 and T2 spermatogonia is useful and convincing.
Possible identification of cells in the migration is also unique.
This is a very dense paper with many claims of discovery but it also comes on the heels of many scRNAseq papers on testis and testis development. Two recent papers on the perinatal period using FAX sorted germ cells are very recent and describe the cell populations and propose lineages. While this current manuscript adds some unique aspects to those papers already published there is a great deal of repetitious approaches and very little attempt to integrate this study with the published work. The authors argue that they also add to understanding the somatic component that the two recent papers did not comment on. The value added by the data on the somatic components is very limited.

With no functional analysis the authors propose two types of spermatogonial stem cells based on gene expression. This is difficult to accept since a stem cell is a stem cell and can give rise to all of the other cells.
Are the S1 and S2 population capable of giving rise to the other cells? Identifying cell populations based solely on gene expression without functional assays has limitations. If S1 and S2 function the same are they different?

Figure S2B presents some distinct challenges including the presence of Stra8 transcripts throughout the germ cell population. Gfra1, an undifferentiated progenitor cell marker appears in the SSc1 population but not in the more developed SSc2

Reviewer 2

*Advance summary and potential significance to field*

The manuscript by Tan et al. explores the transcriptomes of cells consisting perinatal mouse testes at embryonic day (E) 18.5, postnatal day (P) 2, and P7 by single-cell RNA sequencing (scRNA-seq). The authors analyzed 50,859 single cells in total and described their findings both on germ cells and somatic cells.

The authors found that throughout these stages, CDK2A can be used as a pan germ-cell surface marker and that germ cells at E18.5 are all considered to be primary transitional pro-spermatogonia (T1-ProSG) at the mitotic arrest stage. At P2, germ cells were classified into two cell types, intermediate-ProSG (I-ProSG), which are a newly identified population bearing a migratory phenotype and express genes such as Elmo1 and Palld, and secondary transitional-ProSG (T2-ProSG), which show a proliferative phenotype and express genes such as Etv4. At P7, germ cells were classified into three cell types, spermatogonial stem cells-1 (SSC-1), SSC-2, and differentiating

PG (Diff-SG). SSC1 express higher levels of many SSC marker genes, such as Gfra1 and Id4, than SSC2, and were classified further into SSC-1s1 and SSC-1s2, with SSC-1s1 expressing the recently identified SSC markers, Eomes and Cd87. In addition to the SSC markers, SSC-2 express progenitor marker genes, such as Neurog3, Nanos3, and Rarg, and Diff-SG express differentiation markers, such as Kit, Stra8, Dmrb1, and Rhox13. Interestingly, the authors found that Lhx1 and Cd82 can be markers for the most primitive SSC population, SSC-1s1.

The authors also analyzed the somatic cell population and found that Sertoli cells, Leydig cells, and peritubular myoid cells (PTMs) all exhibit dynamic changes of gene expression during the stages examined, with Sertoli cells generating 12 distinct cell clusters, Leydig cells generating 7 distinct cluster, and PTMs generating also7 distinct cluster. Finally, the authors showed potential signaling pathways/interactions between germ cells and somatic cells.

*Comments for the author*

This is a descriptive piece of work that provides useful information regarding potential pathways on how SSCs are specified from ProSG during the perinatal period in mice. Given that there are already several publications with overlapping information, the authors require more rigorous analysis of the data that they acquired.

1. From the analyses provided, it would not be possible for the readers to gain insight into the mechanism for the differentiation of T1-ProSG into I-ProSG to T2-ProSG to SSC1 (s1 and s2) to SSC1 to Diff-SG. The authors need to perform more rigorous analysis to address to this point, e.g., to identify potential signaling pathways and transcription factors that may be involved in these cell-fate transitions.

2. Similarly, although the authors described many distinct clusters for Sertoli cells, Leydig cells, and PTMs, they provided no meaningful analysis on what these differences indicate and how immature cell types gradually mature their phenotypes. The authors need to perform more rigorous analysis to address to these points.

3. The information provided in Figure 5 again does not provide any insightful information regarding the mechanism of the transitions of cellular traits of germ cells and somatic cells during the ~7-day differentiation period.

4. The quality of the scRNA-seq appears to be rather low: mean reads per cell: < 30,000, detected median gene numbers: ≈2,500, low stringent inclusion criteria: 200-6000 genes detected per cell, UMI filter not shown, and therefore, the authors should assess the novel lineage markers and novel clusters more carefully.

For example, cells can be clustered according to their UMI counts rather than their gene expression properties when the UMI counts are highly variable. The authors should examine whether this is not the case for their analysis.

Are the cell clusters that the authors have identified still valid if more stringent criteria are used or if the other published datasets (Velte et al. report P1.5 and P6 transcriptomes, and Hermann et al. and Grive et al. report P6 transcriptomes) are used?

The images shown in Fig. 2F and Fig. 3G are not clear enough, particularly the CD82 staining in Fig. 3G is not convincing. The authors should show images with each channel separately and with wider area to clearly show the specificity of the relevant proteins.

Also, Hermann et al. (2018) has shown that the Id4-GFP+ cells represent a single population at P6, which would be the case at P7. In contrast, the data in Fig. 3B and F indicate that Id4 is expressed not only in SSC-1s1 but also in SSC-1s2 and SSC-2. The authors should clarify this point further. It would be useful if the authors can explore the relationship between Id4-GFP+ cells and CD82 or LHX1-expressing cells by FACS analysis.

5. The authors have compared their transcriptome data with those reported by Grive et al. (2019), concluding that the SSC-1 and SSC-2 subpopulations maintain their states from P6 to the adult, whereas the Diff-SG and SPCs during the first-wave spermatogenesis and in the adults show somewhat different properties. In Fig. S3D, what metrics was used to conclude that Diff-SG were different between the first-wave and the adult spermatogenesis? Also, what do the numbers on the top of the plots indicate? Are they correlation coefficients? Also, the authors should analyze the differentially expressed genes to provide an insight regarding the significance of the differences.

Additional points:
1. As the cells were collected in an unfractionated manner, a table showing the number of the cells collected for each cell type and each time point should be provided
2. Scale bars should be added to the IF figures.
3. The numbers of the cells included in each cluster should be indicated for Fig. 1A, 2A, 2B, and 3A.
4. In Fig. 3E, the numbers of the genes for each cluster should be provided.
5. In Fig. 4B, the order of the panels should be reversed. The developmental stage plot should better be placed on the right as the others.
6. Fig. 5B can be combined in one plot by inverting the row and the column.
7. In Fig. S1B, the dots over the violin plots are too large and hiding the violin plots. Please rearrange this figure, for example, by reducing the sizes of the plots.
8. In Fig. sS2A, please include the number of the samples for each group. This heatmap seems to be created by using less sample numbers compared to the UMAP plots. If so, please describe the filtering criteria.
9. Line 558. typo : for 1”_”h at.

Reviewer 3

*Advance summary and potential significance to field*

In this study, Tan and colleagues explored germ cell and somatic cell heterogeneity during neonatal testis development using the mouse and single cell RNA sequencing analysis. Because both germline and somatic cell development during fetal and neonatal life has historically been understudied in mammals, this manuscript address gaps in knowledge. For the most part, the experimentation is well crafted and appropriately interpreted and the manuscript is clearly written. However, there are some significant deficiencies in my opinion that need to be addressed to bolster validity of the findings and define the advance in knowledge that has been gained.

*Comments for the author*

Unfortunately, the main focus on prospermatogonial development and spermatogonial stem cell genesis using single cell RNA seq lacks novelty in light of recent studies by Law et al., 2019, Nat Comm. and Liao et al., 2019, Development. The Liao et al. studied examined a select subset (i.e. those expressing Oct4-GFP) of postnatal spermatogonia at 5.5 days after birth in mice which is after the prospermatogonial to postnatal spermatogonial transition has occurred and recent studies have indicated that Oct4-GFP+ germ cells in the postnatal testis are primed for differentiation. Thus, in my opinion, the Liao et al. study has limited relevance to studying spermatogonial stem cell genesis from prospermatogonial precursors and should be disregarded in making comparisons to the current study. In contrast, the current study duplicates much of the Law et al. study but provides less depth and breadth of information for prospermatogonia and spermatogonial stem cells (i.e. Law et al. analyzed just germ cells, so the number of reads per cell was greater, and explored more age points). It is surprising that the authors did not compare their dataset to the Law et al. single cell RNA seq dataset for prospermatogonia and postnatal spermatogonia to define similarities and differences. For example, the Law et al. study identified Etv4 as a potential player in prospermatogonial development and the current study also picked up on this gene. This begs the question of what other similarities and likely more interesting what differences there are between the datasets. As written with a focus on prospermatogonia and spermatogonial stem cells, the current study seems to provide incremental advance in knowledge.

The major advance of the current study likely rests with the single cell RNA seq data of somatic cell populations, which the Law et al. study did not examine, but this analysis seems like an

afterthought in the current version of the manuscript. I suggest that the authors retool the manuscript to:
1) make direct comparison to the Law et al. study that defines similarities and differences for germ cells, and 2) highlight the somatic cell datasets which are the first of their kind (unlike the germ cell profiles).

In addition to the lack of novelty by focusing on germ cells, the manuscript also has seemingly other limitations that should be addressed for accuracy:

1) Referring to prospermatogonia or spermatogonia as states brings with it the connotation of a functional ability. The authors have not provided evidence of functional differences between what they are defining as states. In reality, the authors have identified “transcriptome profiles” not functional states and this should be made clear in the manuscript. To simply present the results and discuss them as states implies functional differences but evidence supporting this has not been provided and is misleading to readers. As presented, I find the authors’ labeling of I-ProSG SSC-s1, SSC-s2, and SSC-2 to be ambiguous and lacking of evidence to support a functional difference.

2) The recent study by Law et al., 2019, Nat. Comm. has not been accurately represented by the authors. In that study, the entire germ cell population was profiled throughout late fetal and early postnatal development. In lines 77-86 of the introduction, the authors lump this study in with others that examined adult testes and then make general statements about all of them advancing knowledge of spermatogenesis in adult mice. This is misleading to readers and I feel that the authors need to bring to light in the introduction section that recent studies have already addressed prospermatogonial and spermatogonial heterogeneity by single cell RNA seq profiling during development. Also, in lines 430-432 of the discussion, the authors state that Law et al. FACS sorted Id4-eGFP+ germ cells for scRNA-seq. This is not accurate; the entire prospermatogonial/spermatogonial populations were FACS sorted and profiled, not just the Id4-eGFP+ subset.

3) The authors should revise the statement in the introduction section (lines 44-45) indicating that the extent of prospermatogonial heterogeneity is not clear. The Law et al. study clearly shows heterogeneity of prospermatogonia and at more age points than the current study.

4) The statement made on lines 46-47 about Foxo1 being a well-established prospermatogonia marker should be reconsidered. Foxo1 expression is not specific to the prospermatogonial stage; it is also expressed by postnatal spermatogonial subsets, although the subcellular localization changes.

5) The statement made in lines 190-191 that SSC formation is under way at P7 is not accurate in light of studies by Shinohara et al., 2001, PNAS; Kubota et al., 2004, Biol Reprod; and Law et al., 2019, Nat Comm. Taken together, data from those studies indicate that SSC formation initiates in early development and is completed by P3.

6) The authors claims about identifying a new prospermatogonial subset that converts to a proliferative precursor cell that gives rise to SSCs and a primitive SSC subset that is the direct descendant of prospermatogonia are not substantiated by functional evidence. Stem cells by definition have a unique functional property of being able to regenerate a cellular lineage. Claiming an SSC nature of a cell population must be validated with functional evidence showing the capacity to regenerate or sustain spermatogenesis. The authors have not taken the experimentation to this level, thus the claims of prospermatogonial subsets giving rise to SSCs or a primitive SSC state is anecdotal at present.

7) Morphological studies by Dirk de Rooij reported prospermatogonia along the basement membrane as early as P0 and re-entry to mitosis coincides with prospermatogonia reaching the basement membrane. Thus, T1-prospermatogonia progressively migrate to the basement membrane, resume mitosis, and as a result, become T2. In consideration of these observations, the “I-Prospg” designation made by the authors is confusing. There is asynchrony in the germline throughout development, including PGC specification, gonadal colonization, sex determination, mitotic arrest, etc. so defining these transition periods with subtypes seems to complicate the process, making it more difficult to comprehend a functional difference.

8) The authors subdivided germ and somatic cell populations into various clusters, but rationale for why the number of clusters were selected and whether or not these clusters represent anything biologically relevant, particularly in somatic cell populations, seems to not be clearly defined.

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**First revision**

Author response to reviewers' comments

Point-by-point response to the Reviewers' concerns:

Reviewer 1

We took the liberty of adding numbers to Rev 1's criticisms, below, so they can easily be referred to.
(1) Line 51 "subtle"
Response: We changed "subtly" to "subtle" on line 50 in the revised MS.

(2) This is a very dense paper with many claims of discovery but it also comes on the heels of many scRNAseq papers on testis and testis development. Two recent papers on the perinatal period using FAX sorted germcells are very recent and describe the cell populations and propose lineages. While this current manuscript adds some unique aspects to those papers already published there is a great deal of repetitious approaches and very little attempt to integrate this study with the published work.
Response: Most of the scRNAseq papers that have recently been published are on the subject of the adult testis, which is not relevant to our study. To our knowledge, there are only 2 papers that examined the perinatal time period of testes development (Law et al., 2019; Liao et al., 2019). Liao et al. examined a select spermatogonia subset (germ cells expressing Oct4-GFP) at a specific time point soon after SSC genesis has initiated: P5.5. Since this time point is similar to the P7 time point from our analysis, it provided an opportunity to assess the validity of our analysis. To do this, we combined the Liao et al. scRNAseq dataset with ours and found that the Liao et al. P5.5 germ cell cluster overlapped with our P7 cell cluster (Fig. 1 below; Fig. S3A in the revised MS). In addition, Liao et al. identified CD87 (also named Plaur) as a marker of emergent SSCs at P5.5 (Liao et al., 2019), Consistent with this, we found that Cd87 is mainly expressed in newly emergent undifferentiated SG - what we call the "s1 state" (Fig. S4A in our revised MS). This validates our clustering of emergent SSCs, which we noted on line 190-193 in our revised MS.

Fig. 1. UMAP plot of our scRNAseq dataset combined with the one from Liao et al. (Liao et al., 2019).
Law et al. used scRNAseq analysis to examine germ cells from E16.5 to P6 mice (Law et al., 2019). The germ cells analyzed in their study were Tomato fluorescent protein-positive cells purified by FACS from Blimp1-Cre; tdTomatoflox_STOP_flox; LacZ; Id4-eGfp transgenic mice (their strategy follows from the fact that Blimp-Cre is expressed in PGCs, and thus it drives irreversible expression of tdTomato in descendent germ cells). To determine the relationship of the perinatal germ cell clusters defined by Law et al. with those we defined, we bioinformatically combined their datasets with ours. However, we did not observe a cluster pattern that followed a coherent temporal sequence (Fig. 2, below), even after we re-analyzed the Law et al. raw data with the same sequence alignment tool that we used for our datasets (Fig. 3, below). In addition, a heatmap of differentially expressed genes at different time points from the 2 studies clearly showed that the shifts in gene expression over time do not exhibit a coherent pattern (Fig. 4, below). Sequence depth differences are unlikely to be responsible, as sequence depth is quite similar for the 2 studies, except for the Law et al. E16.5 data, which had higher values (Fig. 5, below).
Fig. 2. Analysis of our dataset combined with that of Law et al. tSNE and UMAP plots of our scRNAseq datasets (the E18.5, P2, and P7 clusters) combined with the Law et al. datasets (the E16, PO, P3, and P6 clusters). Replicates are indicated by the numbers following the hyphen or dot.

Fig. 3. Analysis of our dataset combined with that of Law et al. Top, UMAP plots of our scRNAseq dataset combined with the one from Law et al. Bottom, Trajectory analysis of these combined datasets. Arrow indicates the pseudotime developmental direction.

Fig. 4. Analysis of the Law et al. dataset combined with ours. Left, heatmap of differentially expressed genes (DEGs) from germ cells from the indicated studies and time points. Right, the most statistically enriched GO functions in germ cells from the indicated time points.
Fig. 5. Quality control analysis of the germ cell scRNAseq datasets from Law et al. and our study. The datasets from the two studies were combined and then analyzed in parallel
As an alternative approach, we re-analyzed the Law et al. dataset using the same alignment, filtering, normalization, and clustering parameters used for our dataset. This analysis identified 9 clusters (Fig. 6 below; Fig.

S3B in the revised MS). After identifying the enriched genes expressed from each of these clusters (Table S1), we compared them with the enriched genes expressed from the ProSG subsets that we identified in our study (Fig. 7 below; Fig. S3C in the revised MS). This revealed that C1 and C3 correspond best to the T1-ProSG cluster we defined; C3 and C4 correspond to I-ProSG; and C4 and C5 correspond to T2-ProSG. Consistent with this, the new gene markers we defined for the T1-, I-, and T2-ProSG clusters—Dnmt3l, Kdm1b, Elmo1, Palld, Etv4, and Etv5— preferentially labeled the corresponding Law et al. cell clusters (Fig. 8, below; Fig. S3D in the revised MS). This confirmed the validity of the ProSG subsets markers we identified.
As an independent test of the validity of the cell clusters we defined, we used the well-established stage-specific germ cell markers used by Law et al. for their study (Fig. 4f in Law et al.; Fig. 9, below). This demonstrated that SSC-associated markers (Etv5, Id4, Lhx1, and Ret) initiate expression in the ProSG subsets and maintain expression in the SSC-1 subsets; progenitor markers (Neurog3 and Sox3) are mainly expressed in the more advanced SSC-2 subset; and the differentiation markers (Kit and Stra8) are mainly expressed in the Diff-SG subset (Fig. 10, below; Fig. S3E in the revised MS). We conclude that the comparison of the Law et al. scRNAseq dataset with ours confirms the validity of the germ cell subsets and stage-specific markers we identified.

Fig. 6. Germ cell clusters from purified E16.5 to P6 germ cells studied by Law et al. Their scRNAseq dataset was re-analyzed by us using the UMAP clustering algorithm, as described in the text. Left, clusters names; Right, sample information.

Fig. 7. Venn diagram showing the overlap of genes exhibiting enriched expression in the indicated cell subsets.
C1 to C9 were defined by us from the Law et al. dataset as described in Fig. 6, while T1-, I-, and T2-ProSG are from our study.

Fig. 8. The expression pattern of key ProSG markers that we identified. Their expression is shown on the UMAP plot that we generated from the raw Law et al. datasets from purified E18.5 to P6 germ cells (see Fig. 6).

Fig. 9. Expression of well-established germ cell markers in the ProSG and undifferentiated SG cell subsets we defined. Top, Fig. 4f from Law et al. showing the expression pattern of germ-cell markers used to define their 10 cell clusters. Bottom, Dot plots showing the expression of these germ-cell markers in the 7 germ cell subsets we defined.

Law et al. hypothesized that cluster 1 from E16.5 (“C2” when plotted using our parameters [Fig. 6, above; Fig. S3B in the revised MS]) is the most primitive germ cell subset because it has the highest average expression levels of germline pluripotency markers and stem cell markers, and has the lowest levels of the differentiation-associated markers (Fig. 10, below) (Law et al., 2019). While we agree that cluster C2 is a primitive cell cluster, there is reason to think that many of the other cells from E16.5 (represented as cluster C1 from our analysis of their dataset [Fig. 6, above; Fig. S3B in the revised MS]) are equally if not more primitive than most cells in the C2 cluster. As one line of evidence for this, trajectory analysis found that C2 is more developmentally advanced than C1 (Fig. 11, below). Further evidence is that while the vast majority of C1 cluster cells are mitotically quiescent—a key characteristic of T1-ProSG—many C2 cluster cells are mitotically active (Fig. 12; Fig S3F in our revised MS) - a key characteristic of later stage (T2-ProSG) germ cells (McCarrey, 2013). Despite their apparent differences in maturity and cell-cycle status, the C1 and C2 clusters

are similar molecularly, based on GO and signaling pathway analyses (Fig. 13, below; Table S1 in the revised MS).
Together, these new findings are on lines 194-230 in our revised MS.

Fig. 10. VlnPlots showing the expression of well-established germ-cell marker genes in the C1 and C2 clusters that we defined from the Law et al. dataset (see Fig. 6).

Fig. 11. Monocle trajectory analysis of germ cells from the Law et al. germ cell dataset. Left, cell clusters; Right, time points.

Fig. 12. Cell-cycle gene analysis of germ cells from the Law et al dataset.

Fig. 13. Functions enriched in the Law et al. C1 and C2 clusters. Enriched GO (top) and signaling pathways (bottom) in C1 and C2 cell cluster from Law et al. dataset.

(3) The authors argue that they also add to understanding the somatic component that the two recent papers did not comment on. The value added by the data on the somatic components is very limited.
Response: This is a good point and we thank Rev 1 for this comment. Indeed, to our knowledge, there has been no scRNAseq analysis of somatic cell subsets in the testis during the perinatal period (and only scRNAseq analysis of Sertoli cells in adult testes (Green et al., 2018)). In response to the Reviewer's suggestion, we have done further analysis of the three major testicular somatic cell types—Sertoli cells (SCs), Leydig cells (LCs), and peritubular myoid cells (PTMs)—as summarized below:
(i) We performed detailed analysis of the multiple distinct cell clusters that we identified for each of these 3 cell types over the perinatal period (E18.5 to P7) (Figs. 4, 5, and S6; and Table S2 in the revised MS)
(ii) To ascertain a temporal timeline of the molecular shifts that occur during the development of these 3 somatic cell types, we conducted pseudotime analysis of their patterns of transcriptome changes and associated GO functions during perinatal development (Figs. 4, 5 and S6; Table S2 in the revised MS)
(iii) We identified signaling pathways predicted to be active in these three cell types during the different phases of the perinatal period (Figs. 4, 5 and S6; Table S2 in the revised MS)
A detailed description of these analyses was added to the revised MS on lines 335-424.

(4) With no functional analysis the authors propose two types of spermatogonial stem cells based on gene expression. This is difficult to accept since a stem cell is a stem cell and can give rise to all of the other cells. Are the S1 and S2 population capable of giving rise to the other cells? Identifying cell populations based solely on gene expression without functional assays has limitations. If S1 and S2 function the same are they different?
Response: We apologize if we gave the impression that we showed that s1 and s2 are two types of stem cells. What we showed is these two undifferentiated spermatogonia cell subsets differentially express several genes and they cluster separately, as defined by the UMAP algorithm. With regard to Rev 1's statement that "a stem cell is a stem cell," there is considerable evidence that a given anatomical location does not necessarily only have a single stem cell type. As a case in point, it is well-established that there are two distinct types of stem cell in the olfactory epithelium: (i) globose basal cells, which serve as stem cells for steady-state production of olfactory sensory neurons, and (ii) horizontal basal cells, which serve as reserve stem cells that only become activated by major insults. Thus, by analogy, s1 and s2 could be enriched in active and reserve SSCs, respectively (or vice versa). Consistent with this idea, there is evidence for considerable heterogeneity of SSCs and other types of undifferentiated spermatogonia in mice, based on several lines of evidence (Hermann et al., 2015; Niedenberger et al., 2015; Suzuki et al., 2009; Yoshida et al., 2007). Another possibility is that s1 and s2 are interconvertible cellular states, both of which have SSC activity. Indeed, the notion that mouse undifferentiated spermatogonia and SSCs exist as distinct interconvertible states is supported by past studies (Hara et al., 2014). It is also possible that s1 and s2 have different functions. In this regard, we note that s1 and s2 significantly differ in the profile of signaling and transcriptional factor genes that they express (Fig. S5A and Table S2). To distinguish between these various possibilities, it will be necessary to perform functional experiments, including germ-cell transplantation and lineage-tracing studies.

We have written the following to the revised MS in an attempt to conservatively and accurately describe the s1 and s2 subsets:
In the Results section (lines 245-247), we introduce s1 and s2 by saying: “This separated the SSC-1 subset into two cell clusters that we call “state-1 (s1)” and “s2” (Fig. 3A). Note that by using the word “state,” we are referring to closely related cell subsets that may or may not be interconvertible.”
In the Discussion section (lines 569-574), we describe these two clusters as follows: “We defined these two cell clusters based only on their respective transcriptomes, and thus it remains to be determined whether they have functional differences; e.g., by analogy with stem cells at some other anatomical sites, one of these clusters may be enriched in active SSCs while the other is enriched in reserve SSCs. In support of s1 and s2 having different functions, they differ in their expression of signaling and TF genes (Fig. S5A and Table S1).”
We also say on lines 578-581: “An intriguing possibility is these two states are interconvertible, in alignment with studies suggesting that mouse undifferentiated SG stages are in equilibrium (Hara et al., 2014). In the future, lineage-tracing analysis will be required to definitively ascertain the developmental relationship of the s1 and s2 clusters.”
With regard to function, we defined a marker that labels the s1 cluster and then explained its limitations (lines 602-604): “Determining CD82’s efficacy in purifying SSCs will depend on future germ-cell transplantation experiments (Brinster and Zimmermann, 1994).”

(5) Figure S2B presents some distinct challenges including the presence of Stra8 transcripts throughout the germ cell population. Gfra1, an undifferentiated progenitor cell marker appears in the SSc1 population but not in the more developed SSc2.
Response: We are not entirely clear as to the Reviewer’s question, but it seems he/she is doubtful of the expression pattern of Stra8 and Gfra1. With regard to Stra8, we actually did not find it was broadly expressed; instead it initiates its expression in the SSC-2 cluster (which corresponds to progenitors, based on markers) and reaches its highest level in the Differentiating Spermatogonia (Diff-SG) cluster (Fig. S2C in our revised MS). As an independent means to address this, we re-analyzed scRNAseq raw data from another group (Hermann et al., 2018) that analyzed enriched Id4-eGFPbright, Id4-eGFPdim, and unselected SG cells from P6 testes. UMAP clustering analysis showed that Stra8 mRNA is commonly expressed Id4-eGFPdim cells (which are enriched in progenitors) and rarely expressed in Id4-eGFPbright cells (which are enriched in SSCs) (Fig. 14, below), which is consistent with our findings. As for Gfra1, as indicated by Rev 1, we found it is mainly expressed in cells in the SSC-1 cluster, not the SSC-2 cluster (Fig 3B in our revised MS), which we confirmed through our re-analysis of the Hermann et al. dataset: Gfra1 is mainly expressed in Id4-eGFPbright cells (highly SSC enriched), but rarely in Id4-eGFPdim cells (enriched in progenitors).

Fig. 14. Expression pattern of germ-cell markers in Id4-eGFP+ and Id4-eGFP- early postnatal germ cell populations. (A) UMAP plot showing the cell clusters derived by our re-analysis of the Hermann et al. mouse P6 scRNAseq datasets from the indicated 3 germ cell populations. (B) Expression pattern of the indicated germ cell markers.

Reviewer 2

(1) From the analyses provided, it would not be possible for the readers to gain insight into the mechanism for the differentiation of T1-ProSG into I-ProSG to T2-ProSG to SSC1 (s1 and s2) to SSC1 to Diff-SG. The authors need to perform more rigorous analysis to address to this point, e.g., to identify potential signaling pathways and transcription factors that may be involved in these cell-fate transitions.
Response: For revision, we analyzed our datasets to identify signaling pathways and transcription factors inferred to be active in all 7 of the germ cell stages we identified (Figs. S5A-C in the revised MS; lines 287-307). We agree that this provides insight into the underlying mechanisms by which germ cells progress and differentiate during the perinatal period.

(2) Similarly, although the authors described many distinct clusters for Sertoli cells, Leydig cells, and PTMs, they provided no meaningful analysis on what these differences indicate and how immature cell types gradually mature their phenotypes. The authors need to perform more rigorous analysis to address to these points.
Response: Please see our response to Rev 1, comment 3.

(3) The information provided in Figure 5 again does not provide any insightful information regarding the mechanism of the transitions of cellular traits of germ cells and somatic cells during the ~7-day differentiation period.
Response: Fig. S8 in the revised MS (Fig. 5 of the originally submitted MS) is intended to identify inter-cellular signaling pathways likely to be active during the perinatal period in somatic and germ cells (as measured through expression of ligand-receptor pairs). These signaling pathways between cell subsets have the potential to have a role in the cellular transitions referred to by Rev 2.
To provide more depth with regard to molecular mechanisms important for such cell transitions, we have done the following:
First, we performed the in-depth analysis indicated in our response to Rev 1, comments 2 and 3. This includes extensive comparisons with published relevant datasets and identification of molecular events (e.g., signaling pathways and transcription factors) potentially involved in germ and somatic cell perinatal development (Figs. 4, 5, 6, and S3-7; and Tables S1-2 in the revised MS).
Second, to address the temporal kinetics of cellular signaling mechanisms, we analyzed the dynamics of signaling gene expression during perinatal development in germ cells, Sertoli cells, Leydig cells, and PTM cells (using pseudotime analysis; Table S1 and S2 in the revised MS). We also determined the significantly enriched signaling pathways in each cell type at each stage (Fig. 15, below; Fig 6A in the revised MS).

Fig. 15. Top enriched signaling pathways inferred to be active in germ and somatic cells defined by our study at the indicated time points.

Third, to test the role of signaling empirically, we elected to focus on the Hippo signaling pathway, as this signaling pathway is the statistically most significantly enriched pathway in perinatal germ cells (Fig. 15, above) and is well-known to be essential for a variety of processes, including cell growth, proliferation, differentiation, and migration, in developing organs (Davis and Tapon, 2019; Meng et al., 2016). Using nuclear YAP as a marker of Hippo signaling (Meng et al., 2016), we found that the Hippo pathway is active precisely when ProSG convert into differentiating SG and SSCs. Immunofluorescence analysis showed that the percentage of cells with nuclear YAP increases from <5% to >95% between P0 and P2; the brightness of nuclear YAP staining reaches its highest level at P3; and drops to nearly 0 by P7 (Fig. 16, below; Figs. 6C and S7 in the revised MS). This dramatic burst of nuclear-localized YAP coinciding with the generation of both differentiating SG and SSCs raises the possibility that Hippo signaling has roles in one or both of these cell transition events.

Fig. 16. Evidence that Hippo signaling is active during the ProSG-to-SG transition. Top, immunofluorescence analysis of early postnatal (P0, P2, P3, P5, P6, and P7) testes sections co-stained with antisera against YAP and TRA98. Bottom left, quantification of the percentage of germ cells (labeled with TRA98) that harbor nuclear YAP. Dim and Bright nuclear YAP staining were distinguished using the imageJ program, with a cut-off of 50% between bright and dim. Of note, the bright positive signals outside of the tubule are blood and endothelial cells. Bottom right, number of cells counted.
Finally, we determined the temporal expression of signaling receptor-ligand pairs during perinatal development. As an example, this analysis revealed that Gdnf, which encodes the GDNF signaling ligand, is expressed in E18.5 Sertoli cells, while Gfr$\alpha$1 and Ret, which encode the GDNF signaling receptors, do not initiate expression until after birth (Fig. 17A, below; Fig. 6E in the revised MS), suggesting that GDNF signaling is not active in germ cells until after birth (GDNF may have non-germ cell roles before birth). As another example, Bmp2 and Bmpr1a, which encode a BMP signaling ligand and a BMP receptor, respectively, both initiate expression between E18.5 and P2 (Fig. 17B, below; Fig. 6F in the revised MS). Because the co-expression of the ligand and receptor is induced in different cell types, this suggests coordinated gene regulation to mediate BMP signaling between Leydig cells and ProSG in a temporally specific manner.
Together, the above lines of evidence strongly suggest that both inter- and intra-cellular signaling pathways are widely used in germ and somatic cells during the perinatal period.

Fig. 17. Violin plots showing the expression pattern of ligand-receptor pairs in the indicated cell types and stages. (A) GDNF signaling components. (B) BMP signaling components.

(4) The quality of the scRNA-seq appears to be rather low: mean reads per cell: < 30,000, detected median gene numbers: ≈2,500, low stringent inclusion criteria: 200-6000 genes detected per cell,

UMI filter not shown, and therefore, the authors should assess the novel lineage markers and novel clusters more carefully. For example, cells can be clustered according to their UMI counts rather than their gene expression properties when the UMI counts are highly variable. The authors should examine whether this is not the case for their analysis. Are the cell clusters that the authors have identified still valid if more stringent criteria are used or if the other published datasets (Velte et al. report P1.5 and P6 transcriptomes, and Hermann et al. and Grive et al. report P6 transcriptomes) are used?
Response: We are sorry that these parameters were not clearly described in our originally submitted MS. Both mean reads per cell and detected median gene numbers were generated by CellRanger before normalization and filtering. For our clustering analysis of total testicular cells, we used a cut-off of 200-6000 expressed genes detected per cell. For our subsequent clustering analysis of each cell type, we used a cut-off of 1000-6000 expressed genes detected per cell; other parameters were 2500< UMI<40000, percent mito <0.15. We have added this information to the revised Materials & Methods. When we compared our datasets with other published 10X genomics scRNAseq datasets (from the three studies that the Reviewer mentioned), we found that the sequence depth of our datasets is quite similar or even better than many of the other datasets (Fig. 18, below).
Fig 18. Violin plots showing nFeature_RNA, nCount_RNA, and mitochondrial gene expression profiles from the indicated studies.
As Rev 2 suggested, we also clustered cells on the basis of UMI counts. This gave rise to clusters similar to those generated based on genes expression (compare Fig. 19, below, with Fig 2A in the revised MS).
Further confidence in our clustering analysis was its verification by comparison with scRNAseq datasets from Law et al., Liao et al., and Hermann et al., as indicated in our response to Rev 1, comments 2 and 5.

Fig 19. UMAP plot of the germ cells from our dataset clustered based on the UMI values in each cell.

(5) The images shown in Fig. 2F and Fig. 3G are not clear enough, particularly the CD82 staining in Fig. 3G is not convincing. The authors should show images with each channel separately and with wider area to clearly show the specificity of the relevant proteins.
Response: We apologize for the quality of the images in Figs. 2F and 3G. In the case of Fig. 2F, the image quality was compromised during the pdf conversion. Below, we provide an enlarged image of Fig. 2F (reproduced as Fig. 20, below), which we hope will be found to be acceptable.

Fig. 20. IF analysis of P2 and P3 testes sections stained with the antibodies shown. The sections were co-stained with an antibody against KI-67. Scale bar, 20 μm.

As for Fig. 3G, the signal with the antisera we originally used against CD82 staining was not specific for germ cells. We considered the possibility that this was due non-specific binding by this antisera, so we tried another CD82 antisera (cat# LS-C100497-400), This CD82 antisera clearly stained germ cells, but it also stained the cytoplasm of Sertoli cells (Fig. 21, below; Fig. S4F in the revised MS). This is consistent with our scRNAseq data, which showed that Cd82 mRNA is expressed in some Sertoli cells. This Sertoli cytoplasmic staining makes it difficult to convincingly quantify germ cell CD82 staining, as the cytoplasm of Sertoli cells is large and each Sertoli cell is bound by many germ cells. As an alternative approach, we performed FACS analysis with the new CD82 antisera on dissociated testicular cells from P7 transgenic mice expressing the Id4-eGFP SSC marker. This analysis showed that CD82+cells are largely Id4-eGFPbright cells, indicative of CD82 labeling SSCs (Fig. 21, below; Fig. 3H in the revised MS). Since Id4-eGFPbright cells are regarded as the most primitive germ cell population at early postnatal time points (Helsel et al., 2017), this also supports our contention that CD82+ cells are the most primitive emergent undifferentiated spermatogonia.

Fig. 21. CD82 specifically labels a subset of postnatal Id4-eGFP+ cells. Top, IF analysis of P7 Id4-eGFP testis sections stained with a CD82 antisera. Scale bar, 20 μm. Bottom right, FACS plot of P7 mouse Id4-eGFP testicular cells stained with the same CD82 antisera used for IF analysis. The percentage of positive cells are indicated. Bottom left, negative control (Ctrl) stained with only the 2nd antisera (anti-rabbit Ig-PE).

(6) Also, Hermann et al. (2018) has shown that the Id4-GFP+ cells represent a single population at P6, which would be the case at P7. In contrast, the data in Fig. 3B and F indicate that Id4 is

expressed not only in SSC-1s1 but also in SSC-1s2 and SSC-2. The authors should clarify this point further. It would be useful if the authors can explore the relationship between Id4-GFP+ cells and CD82 or LHX1-expressing cells by FACS analysis.
Response: Rev 2 makes a good point regarding Id4 expression. Note, however, that Hermann et al. measured the expression of an Id4-eGFP transgene, whereas we measured endogenous Id4 expression. Given that the 17 kb Id4 transgene in Id4-eGFP transgenic mice resides in a non-natural position in the genome and may not contain all Id4 regulatory elements, it would not be surprising if this transgene exhibited some differences in expression from the endogenous Id4 gene. We also note that our clustering analysis showed that endogenous Id4 is most commonly expressed in SSC-s1, with less cells expressing Id4 in SSC-s2, and very few cells expressing Id4 in SSC-2 (Figs. 3B and F in the revised MS). This finding is consistent with SSC-s1 being more enriched in SSCs than SSC-s2, and supports our contention that SSC-2 cells are predominantly progenitors and have few SSCs. With regard to Rev 2's question about the relationship between Id4-GFP+ and CD82- and LHX1-expressing cells, we addressed this by re-analyzing the Hermann et al. P6 scRNAseq dataset from enriched Id4-eGFPbright, enriched Id4-eGFPdim, and unselected SG cells. This analysis showed Lhx1 and Cd82 are expressed in a subset of Id4-eGFPbright cells (Fig 22, below; Fig. S4E in the revised MS). This provides evidence that Lhx1 and Cd82 mark SSCs. To further test this possibility, we performed FACS analysis of CD82 expression on testicular cells from P7 Id4-eGFP mice. This showed that virtually all CD82+ are Id4-eGFPbright cells (Fig. 21, above; Fig. 3H in the revised MS), confirming what we observed from the Hermann et al. scRNAseq dataset. Some Id4-eGFPbright cells lack CD82 expression, confirming that CD82 marks a specific subset of Id4-eGFPbright cells.

Fig. 22. Cd82 and Lhx1 are expressed in Id4-eGFPbright cells. (A) UMAP plot of our re-analysis of the Hermann et al. scRNAseq dataset of the indicated P6 germ cell populations. (B) Expression pattern of the indicated genes.

(7) The authors have compared their transcriptome data with those reported by Grive et al. (2019), concluding that the SSC-1 and SSC-2 subpopulations maintain their states from P6 to the adult, whereas the Diff-SG and SPCs during the first-wave spermatogenesis and in the adults show somewhat different properties. In Fig. S3D, what metrics was used to conclude that Diff-SG were different between the first-wave and the adult spermatogenesis? Also, what do the numbers on the top of the plots indicate? Are they correlation coefficients? Also, the authors should analyze the differentially expressed genes to provide an insight regarding the significance of the differences.
Response: We apologize that this information was not clearly described. The numbers on the top of the plots are indeed correlation coefficients, and we used them to show differences in the subsets (Figs. S5D and E in the revised MS). We provided the list of differentially expressed genes between first-wave Diff-SG and Diff-SG in subsequent waves of spermatogenesis in Table S1.

Additional points:

(8) As the cells were collected in an unfractionated manner, a table showing the number of the cells collected for each cell type and each time point should be provided
Response: This information is provided in Table S4 in the revised MS.

(9) Scale bars should be added to the IF figures.
Response: Scale bars have been added to Figs. 2C, 2D, 2F, 3G, S4F, and S7.

(10) The numbers of the cells included in each cluster should be indicated for Fig. 1A, 2A, 2B, and 3A.
Response: The cell number are now indicated in the figure legends of Figs. 1-3.

(11) In Fig. 3E, the numbers of the genes for each cluster should be provided.
Response: The gene numbers are now indicated in Fig. 3E.

(12) In Fig. 4B, the order of the panels should be reversed. The developmental stage plot should better be placed on the right as the others.
Response: The panel order has been reversed in Fig. 4C (Fig. 4B in the originally submitted MS).

(13) Fig. 5B can be combined in one plot by inverting the row and the column.
Response: Fig. 6D (Fig. 5B in the originally submitted MS) has been altered as suggested.

(14) In Fig. S1B, the dots over the violin plots are too large and hiding the violin plots. Please rearrange this figure, for example, by reducing the sizes of the plots.
Response: This figure has been rearranged as suggested.

(15) In Fig. S2A, please include the number of the samples for each group. This heatmap seems to be created by
using less sample numbers compared to the UMAP plots. If so, please describe the filtering criteria.
Response: We used the same number of cells to make the heatmap (in Fig. S2A) as for the UMAP plots (in Fig. 2A). This was accomplished using the “DoHeatmap” function in Seurat.

(16) Line 558. typo : for 1”_”h at.
Response: This was fixed.

Reviewer 3

Unfortunately, the main focus on prospermatogonial development and spermatogonial stem cell genesis using single cell RNA seq lacks novelty in light of recent studies by Law et al., 2019, Nat Comm. and Liao et al., 2019, Development. The Liao et al. studied examined a select subset (i.e. those expressing Oct4-GFP) of postnatal spermatogonia at 5.5 days after birth in mice which is after the prospermatogonial to postnatal spermatogonial transition has occurred and recent studies have indicated that Oct4-GFP+ germ cells in the postnatal testis are primed for differentiation. Thus, in my opinion, the Liao et al. study has limited relevance to studying spermatogonial stem cell genesis from prospermatogonial precursors and should be disregarded in making comparisons to the current study. In contrast, the current study duplicates much of the Law et al. study but provides less depth and breadth of information for prospermatogonia and spermatogonial stem cells (i.e. Law et al. analyzed just germ cells, so the number of reads per cell was greater, and explored more age points). It is surprising that the authors did not compare their dataset to the Law et al. single cell RNA seq dataset for prospermatogonia and postnatal spermatogonia to define similarities and differences. For example, the Law et al. study identified Etv4 as a potential player in prospermatogonial development and the current study also picked up on this gene. This begs the question of what other similarities and likely more interesting what differences there are between the datasets. As written with a focus on prospermatogonia and spermatogonial stem cells, the current study seems to provide incremental advance in knowledge.
Response: We agree that a detailed comparison with Law et al. is important. We did this, as explained in response to Rev 1, Point 2. We also agree that Liao et al. is less relevant to our study, but nonetheless their scRNAseq dataset was useful in validating one of the time points in our scRNAseq dataset, as also explained in our response to Rev 1, Point 2.

The major advance of the current study likely rests with the single cell RNA seq data of somatic cell populations, which the Law et al. study did not examine, but this analysis seems like an afterthought in the current version of the manuscript. I suggest that the authors retool the manuscript to make direct comparison to the Law et al. study that defines similarities and differences for germ cells, and 2) highlight the somatic cell datasets which are the first of their kind (unlike the germ cell profiles).
Response: Please see our response to Rev 1, comment 3.

(1) Referring to prospermatogonia or spermatogonia as states brings with it the connotation of a functional ability. The authors have not provided evidence of functional differences between what they are defining as states. In reality, the authors have identified “transcriptome profiles” not functional states and this should be made clear in the manuscript. To simply present the results and discuss them as states implies functional differences but evidence supporting this has not been provided and is misleading to readers. As presented, I find the authors’ labeling of I-ProSG, SSC-s1, SSC-s2, and SSC-2 to be ambiguous and lacking of evidence to support a functional difference.
Response: We largely addressed these issues in our response to Rev 1, comment 4, above. Our reply to that question was with regard to the SSC1-s1 and -s2 cell clusters, but our answer applies generally to all the cell clusters we studied. With regard to the names we have given to the cell clusters we identified, we believe these names are logical, and we explain their origin when they are first brought up. With regard to function, while we agree that the cell clusters were defined

only on the basis of their transcriptomes, in our revised MS we made a large effort to ascribe possible physiological functions for them through in-depth analysis of their enriched signaling and transcription factor genes (see responses to other questions in this rebuttal letter). We also provided direct empirical evidence that the Hippo signaling pathway is active in the cell clusters associated with the transition between pro-spermatogonia and spermatogonia (see Fig. 16, above).

(2) The recent study by Law et al., 2019, Nat. Comm. has not been accurately represented by the authors. In that study, the entire germ cell population was profiled throughout late fetal and early postnatal development. In lines 77-86 of the introduction, the authors lump this study in with others that examined adult testes and then make general statements about all of them advancing knowledge of spermatogenesis in adult mice. This is misleading to readers and I feel that the authors need to bring to light in the introduction section that recent studies have already addressed prospermatogonial and spermatogonial heterogeneity by single cell RNA seq profiling during development.
Response: We apologize for underrepresenting the Law et al. paper. In the Introduction of our revised MS, we now say: “using germ cells purified from transgenic reporter mice, recent scRNAseq studies have studied some of the events that proceed spermatogenesis, including SSC establishment (Law et al., 2019; Liao et al., 2019)” (lines 81- 84). We provide more detail about the Law et al. study in the section in the Results where we perform comparative analysis with their datasets. We introduce their study in the Results section in the following way: “Law et al. used scRNAseq analysis to examine germ cells from E16.5 to P6 mice (Law et al., 2019). The germ cells analyzed in their study were Tomato fluorescent protein-positive cells purified by FACS from Blimp1-Cre; tdTomatoflox_STOP_flox; LacZ; Id4-eGfp transgenic mice (their strategy follows from the fact that Blimp-Cre is expressed in PGCs, and thus it drives irreversible expression of tdTomato in descendent germ cells)” (lines 194-198).
Also, in lines 430-432 of the discussion, the authors state that Law et al. FACS sorted Id4-eGFP+ germ cells for scRNA-seq. This is not accurate; the entire prospermatogonial/spermatogonial populations were FACS sorted and profiled, not just the Id4-eGFP+ subset.
Response: We apologize for this mistake. In our revised MS, we corrected this statement in lines 194-198. However, it should be noted that Law et al. did not necessarily examine “the entire prospermatogonial / spermatogonial populations,” as they did not examine unselected cells. They FACS-sorted cells that expressed CRE from the Blimp1 promoter to select for germ cells. This elegant approach likely marked most germ cells, but it cannot be excluded that some germ cells failed to express sufficient CRE from the Blimp1 promoter to be marked with GFP.

(3) The authors should revise the statement in the introduction section (lines 44-45) indicating that the extent of prospermatogonialheterogeneity is not clear. The Law et al. study clearly shows heterogeneity of prospermatogonia and at more age points than the current study.
Response: We deleted this statement.

(4) The statement made on lines 46-47 about Foxo1 being a well-established prospermatogonia marker should be reconsidered. Foxo1 expression is not specific to the prospermatogonial stage; it is also expressed by postnatal spermatogonial subsets, although the subcellular localization changes.
Response: We believe our original statement that cytoplasmic FOXO1 is a ProSG marker is correct. While FOXO1 is also present in spermatogonia recently generated from ProSG, it is reported to be nuclear in this context (Goertz et al., 2011), a claim that we have verified (Song et al., 2016). Our aim is not to suggest that FOXO1 is a perfect ProSG marker; only to acknowledge that it was a previously identified ProSG marker that has some utility.

(5) The statement made in lines 190-191 that SSC formation is under way at P7 is not accurate in light of studies by Shinohara et al., 2001, PNAS; Kubota et al., 2004, Biol Reprod; and Law et al., 2019, Nat Comm. Taken together, data from those studies indicate that SSC formation initiates in early development and is completed by P3.
Response: By “underway,” we meant that SSC formation has already initiated and is ongoing. We have rephrased this sentence to say: “At P7, soon after the first SSCs emerge ...” (lines 234-235).

(6) The authors claims about identifying a new prospermatogonial subset that converts to a proliferative precursor cell that gives rise to SSCs and a primitive SSC subset that is the direct descendant of prospermatogonia are not substantiated by functional evidence. Stem cells by

definition have a unique functional property of being able to regenerate a cellular lineage. Claiming an SSC nature of a cell population must be validated with functional evidence showing the capacity to regenerate or sustain spermatogenesis. The authors have not taken the experimentation to this level, thus the claims of prospermatogonial subsets giving rise to SSCs or a primitive SSC state is anecdotal at present.
Response: We apologize if we gave the impression that we had definitively ascertained the developmental relationship of the cell populations we analyzed. We had intended to make it clear that the developmental relationships we discussed were only a model based on evidence. In our revised MS, we edited the text in an attempt to clearly explain that our study identified these cell populations based on single-cell transcriptome profiles, not functional assays. For example, in the Abstract, we said: “Using single-cell RNA sequencing, we identified 3 temporally distinct ProSG cell subsets...” (line 6). In the Discussion, we said: “Through scRNAseq analysis, we identified...” (line 487); “We defined these two cell clusters based only on their respective transcriptomes, and thus it remains to be determined whether they have functional differences; e.g., by analogy with stem cells at some other anatomical sites, one of these clusters may be enriched in active SSCs while the other is enriched in reserve SSCs...” (line 569-572); “Determining CD82’s efficacy in purifying SSCs will depend on future germ-cell transplantation experiments (Brinster and Zimmermann, 1994).” (line 602-604).

(7) Morphological studies by Dirk de Rooij reported prospermatogonia along the basement membrane as early as P0 and re-entry to mitosis coincides with prospermatogonia reaching the basement membrane. Thus, T1- prospermatogonia progressively migrate to the basement membrane, resume mitosis, and as a result, become T2. In consideration of these observations, the “I-Prospg” designation made by the authors is confusing. There is asynchrony in the germline throughout development, including PGC specification, gonadal colonization, sex determination, mitotic arrest, etc. so defining these transition periods with subtypes seems to complicate the process, making it more difficult to comprehend a functional difference.
Response: We were aware of this study by Dirk de Rooij but did not refer to it because of space constraints (it has been added to the revised MS). Of note, there are also several other papers on this topic from both mice and rats. There is some disagreement in these papers as to the timing of ProSG mitotic re-initiation and migration to the basement membrane, a topic that is beyond the scope of our study to discuss. What we have done is add the following to the Introduction: “During the time interval that this T1-to-T2-ProSG conversion event occurs, these transitioning germ cells undergo progressive migration from the center of the seminiferous tubule to its periphery (Kluin and de Rooij, 1981; McGuinness and Orth, 1992). This is the site where SSCs are thought to first form from T2-ProSG, and it is also...” (lines 27-31).
With regard to I-ProSG, our clustering and trajectory analysis clearly defined this cell cluster as a distinct cell subset that is developmentally inbetween the well-established T1- and T2-ProSG subsets. We provided both cellular and molecular evidence that I-ProSG are migratory. That said, we do not claim they are exclusively migratory, nor do we claim that other ProSG subsets cannot be migratory. Indeed, in the Discussion, we suggest that, in some cases, I-ProSG may be a priming stage prior to actual migration to the basement membrane. Thus, we believe we have been appropriately conservative in our assessment of this subset. Our new description of I-ProSG is on lines 151-156 and 184-186 in the Results section and on lines 525-560 in the Discussion section.
We respectively disagree that defining new cell subtypes will make it more difficult to understand biological and development events. Identifying new cell subsets can reveal key transition points during development. We believe this is the case with the I-ProSG subset we identified. Indeed, we explain on lines 540-548 in the Discussion section that the identification of this new subset may be useful in determining whether there is a mechanistic link between ProSG migration and mitotic re-initiation. Identifying new cell subsets can also reveal new functional cell types. Regardless of the eventual outcome, we believe that being able to define distinct cell subsets or states through scRNAseq analysis will typically clarify (rather than obscure) biological meaning.

(8) The authors subdivided germ and somatic cell populations into various clusters, but rationale for why the number of clusters were selected and whether or not these clusters represent anything biologically relevant, particularly in somatic cell populations, seems to not be clearly defined.
Response: We clustered both somatic and germ cells using UMAP clustering, an approach based on the K-nearest neighbor graph. We chose this approach as it is reported to provide the highest reproducibility and the most meaningful organization of cell clusters, when compared with other clustering tools (Becht et al., 2018). In the case of somatic cells, we found that many of the UMAP-

defined clusters were very close to each other, and thus, in preparation for this revision, we merged these similar clusters, based on their enriched gene expression (using Pearson's correlation analysis), as well as their developmental stage and cell-cycle status. This allowed us to reduce the Sertoli cell, Leydig cell, and PTM clusters from 12, 7, and 7, respectively, to 6, 3, and 3, respectively (see Figs. 4, 5, and S6 in our revised MS). We believe these "minimal clusters" are more logical and biologically relevant than the large number of somatic cell clusters we previously defined. We thank Rev 3 for pointing out this issue.

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Second decision letter

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MS TITLE: Pro-spermatogonia development and spermatogonial stem cell genesis defined at single-cell resolution

AUTHORS: Kun Tan, Hye-Won Song, and Miles Wilkinson

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. Reviewers 1 and 2 have several suggestions to improve the clarity of the paper. Reviewer 3 remains concerned about how previous published work is described. It would be helpful for readers if you expand your summary of the work of Law et al. and that you mention the analysis of somatic cell populations in the Abstract. In addition, the suggestion to make clear that inferences of cell identity are based on the transcriptome of the profiled cells seems appropriate.

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*

I congratulate the authors on their major revisions of this manuscript. They have carefully answered the reviewers concerns and integrated their results with the Law and Liao studies. The major advance in this manuscript is the discovery of an additional pro-spermatogonial subtype. While this new subtype is defined only by transcriptional evidence it adds to our knowledge of pro-spermatogonial development.

*Comments for the author*

Most of my prior concerns were addressed in this revision. I feel that this is now a solid manuscript ready for publication. The only remaining concern is Fig 7 that shows the model for their data. In this figure and elsewhere in the paper the term used is SSC-s1 or SSC-s2. This may be appropriate in parts of the discussion but the Fig 7 which lacks discussion these seem to represent progenitor undifferentiated spermatogonia. The term SSC should be reserved for stem cell populations alone.

Reviewer 2

*Advance summary and potential significance to field*

The revised manuscript by Tan et al. has improved significantly from the former version, by responding to most of the reviewerÂ’s comments, including the addition of comparative analysis with public datasets and comments to the development of somatic cells and to the signaling ligand-receptor pairs along testis development. It has now become a useful resource for readers in this field.

*Comments for the author*

A couple of minor points should be addressed before publication.

1. Dnmt3l has been already shown to be expressed in the prospermatogonia of fetal and newborn mice (Sakai et al., 2004) and functional studies using KO mice have been reported from a couple of labs. The descriptions in lines 146-152 and 518-521 sound like this manuscript is the first to detect Dnmt3l at T1-SPGs. The authors should better refer to these papers and describe that the finding in this manuscript is 'in line with' these reports.

2. The nuclear localization of YAP at P2-3 is interesting and may reflect the transient loss of Hippo activity at this stage. The authors may refer to the recent paper of germ cell specifc YAP conditional knockout mouse in the discussion (Abou Nader et al., 2019).

3. The cell numbers should better be added to the legends of Fig 4 and 5.

4. In Fig 6D, it has become space efficient by making in one row, but some of the gene names are overlapped. It is easier to read when arranged like Fig S4A. Also, grouping as in Fig S3E might be helpful for intuitive understanding of the figure. Another option may be using different color coding between ligands and receptors as in Fig S8, however, not necessarily and leave this to the authors' decision.

5. Please double check the figure numbers throughout the text. I found some mismatches. In the section starting from P13 line 315, the figures seem to be S5 and not S4?

Sakai Y, Suetake I, Shinozaki F, Yamashina S, Tajima S. (2004) Co-expression of de novo DNA methyltransferases Dnmt3a2 and Dnmt3L in gonocytes of mouse embryos. Gene Expr Patterns; 5(2):231-7.
Abou Nader N, Levasseur A, Zhang X, Boerboom D, Nagano MC, Boyer A. (2019) Yes-associated protein expression in germ cells is dispensable for spermatogenesis in mice. Genesis; 57(10):e23330. doi: 10.1002/dvg.23330.

Reviewer 3

*Advance summary and potential significance to field*

The information gained by comparing scRNAseq profiles to that of Law et al. 2019, Nat Comm is an important advance to the field. In addition, the scRNAseq profiles of somatic cell populations in perinatal testes have not been previously reported and are a major advance in knowledge.

*Comments for the author*

In the revised version, the authors have addressed some of my major concerns but others have not been sufficiently attended to.

First, in light of previously published studies, the title, summary statement, and abstract of the current manuscript should be revised. The title and summary statement imply that prospermatogonial development and spermatogonial stem cell genesis has not been profiled at the single cell level previously. However, this has already been accomplished by studies of Law et al., 2019, Nat Comm., which profiled fetal and neonatal prospermatogonia at more time points and with more total cells than the current study. The title and summary statement don't reflect novel advances that are made by the current study; in particular the studying of somatic cell populations in perinatal testes, which has not been reported previously, is left out completely. Also, Law et al. already described three different prospermatogonial subsets that emerge through developmental time, thus the authors statement in the abstract of the same discovery is redundant of existing knowledge and should be revised appropriately.

Previous studies that already profiled prospermatogonial and spermatogonial stem cell development still have not been appropriately represented in the introduction section. The authors' experiments largely duplicate the Law et al. study which is fine but the gaps in knowledge not filled by that study which are being addressed by the current study should be defined. Rather, the the statement "recent scRNAseq studies have studied some of the events that proceed spermatogenesis, including SSC establishment" has been inserted to acknowledge Law et al. which

is not really accurate because prospermatogonia were profiled throughout development time and at a greater number of development age points than the current study. It would be appropriate for the authors to add a few sentences that accurately reflect previous studies that have examined prospermatogonial development with scRNAseq and then define what the knowledge gaps are that their studies have addressed.

The authors' statement on lines 239-240 that SSCs emerge at P7 in mice needs to be reconsidered. If one is going define SSCs functionally based on the capacity to generate colonies of spermatogenesis, they emerge much earlier. Studies of Kubota et al., 2004, Biol. Reprod showed that cells in a P0 mouse testis can form spermatogenic colonies after transplant and Law et al., 2019, Nat Comm showed that some even exist in testes at E18.5.

There is still lack of functional endpoints to support the authors' claims of identifying different prospermatogonial and SSC subsets. The data presented is descriptive and certainly suggestive but without a level of experimentation that assesses functional differences, the authors' conclusions are too strong and somewhat misleading to readers outside of the field. For example:

The abstract should be revised to indicate that the identified spermatogonial subsets are based on transcriptome signatures. As written, as reader of the abstract alone could be left with an impression that these subsets were defined based on functional differences.

Lines 586-592: the statement made about the most naïve nature of a spermatogonial subset needs to be clarified with wording that this is at the transcriptome level but functional assessment is lacking. In addition, the statement about a possible interconvertable nature of undifferentiated spermatogonial subsets needs to be qualified with wording that functional endpoints are lacking to support this. Moreover, stating that lineage tracing will be needed to ascertain developmental relationships does not necessarily imply that functional distinctions will be gained.

Overall, the authors should better define what is meant by "SSC heterogeneity" as presented on line 580 and SSC states 1, s1, s2, and 2 as presented in Figures 2 and 7. If SSC is a functional definition of spermatogonia that support continual spermatogenesis or regenerate spermatogenesis after transplant, what is heterogeneous about this or how are different states defined? Heterogeneity in the expression level of certain genes may or may not correspond to a functional difference. In the absence of functional data, SSC subsets identified in the current study should be better described as being based on transcriptome heterogeneity. As presented, the connotation is functional differences.

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**Second revision**

Author response to reviewers' comments

We thank the reviewers for their positive comments, as well as their suggestions for further improving our manuscript. Below, we provide our point-by-point response. In addition to editing the manuscript in accordance with the reviewers' suggestions, we cut its length from 9,541 to 7,600 words to conform to the editorial policy of Development.

Reviewer 1

The only remaining concern is Fig 7 that shows the model for their data. In this figure and elsewhere in the paper the term used is SSC-s1 or SSC-s2. This may be appropriate in parts of the discussion but the Fig 7 which lacks discussion these seem to represent progenitor undifferentiated spermatogonia. The term SSC should be reserved for stem cell populations alone.
Response: We thank Rev 1 for this comment. We agree that the term "SSC" should be reserved only for describing stem cells.

We have added the following to the Fig. 7 legend: 'Note that the cell clusters with the "SSC" designation contain undifferentiated SG that express SSC marker genes but whose SSC functional activity remains to be tested.'
We also revised the text in the Results section describing SSC-related cell clusters (lines 175-188) as follows: '"SSC-1" and "SSC-2" are comprised mainly of undifferentiated SG, based on expression of known undifferentiated SG markers (Figs. 2B and S2C). SSC-1 express higher levels of many SSC marker genes (e.g., Gfr$\alpha$1 and Id4) than SSC-2 (Fig. S2C), suggesting that SSC-1 are more primitive than SSC-2. In contrast, SSC-2 express higher levels of progenitor marker genes (e.g., Neurog3, Nanos3, and Rarg) than SSC-1 (Fig. S2C). While we did not perform a functional assay to ascertain the function of cells in the SSC-1 and -2 clusters, their expression pattern suggests that SSC-1 and SSC-2 are enriched for SSCs and progenitors, respectively. Re-clustering of the P7 germ cells separated the SSC-1 subset into two cell clusters that we named SSC-s1 and SSC-s2 (Fig. 3A), which differentially express several genes (Fig. 3B; Table S1). SSC-s1 expresses the recently identified SSC markers, Eomes and Cd87 (also known as Plaur) (Fayomi and Orwig, 2018; Liao et al., 2019) (Fig. S4A; Table S1), raising the possibility that SSC-s1 is the more primitive of these two cell clusters, a possibility confirmed by Monocle pseudotime analysis (Figs. 3C, D).'
We also revised what we wrote about this topic in the Discussion (lines 424-431): 'We found that the SSC-s1 cell cluster is the most naïve, based on Monocle psuedotime trajectory analysis and the expression pattern of known SSC markers (i.e., Eomes and Plaur) and SSC markers identified in this study (e.g., Lhx1 and Cd82). While the functional significance of this difference in gene expression is not known, one possible explanation is that SSC-s1 and SSC-s2 differ in their endurance as stem cells. Given that SSC-s1 is more primitive than SSC-s2, it may contain cells that tend to retain SSC activity for longer than SSC-s2 cells. Another possibility is that SSC-s1 and SSC-s2 are interconvertible states, in alignment with studies suggesting that mouse undifferentiated SG stages are in equilibrium (Hara et al., 2014).'

Reviewer 2

1.Dnmt3l has been already shown to be expressed in the prospermatogonia of fetal and newborn mice (Sakai et al., 2004) and functional studies using KO mice have been reported from a couple of labs. The descriptions in lines 146-152 and 518-521 sound like this manuscript is the first to detect Dnmt3l at T1-SPGs. The authors should better refer to these papers and describe that the finding in this manuscript is 'in line with' these reports.
Response: Rev 2 makes a good point regarding DNMT3L. We were aware of Sakai et al. 2004 but did not cite it because of space constraints and because this paper only showed that DNMT3L is expressed in ProSG; it did not define DNMT3L as a T1-ProSG marker. In the revised MS, we cite Sakai et al. 2004, as well as papers describing the consequences of Dnmt3l loss (lines 108-116, 372-375).

2.The nuclear localization of YAP at P2-3 is interesting and may reflect the transient loss of Hippo activity at this stage. The authors may refer to the recent paper of germ cell specifc YAP conditional knockout mouse in the discussion (Abou Nader et al., 2019).
Response: We thank Rev2 for bringing this recent paper to our attention. The authors found that conditionally knocking out Yap in germ cells did not cause any detectable phenotypic defects, including in spermatogenesis. One explanation for this is that Hippo signaling is dispensable for mouse spermatogenesis. However, another possibility is that the Yap paralog, Taz (Meng et al., 2016), provides Hippo signaling function in the absence of Yap, a possibility that would only be revealed by conditional knockout of both Yap and Taz. We favor the latter hypothesis given the dramatic burst of Hippo signaling activity we observed specifically during the ProSG-to-SSC transition. In the revised MS, we cited the Nader et al. paper and briefly discussed the above issues in the Discussion (lines 488-494).

3.The cell numbers should better be added to the legends of Fig 4 and 5.
Response: We have done this.

4.In Fig 6D, it has become space efficient by making in one row, but some of the gene names are overlapped. It is easier to read when arranged like Fig S4A. Also, grouping as in Fig S3E might be helpful for intuitive understanding of the figure. Another option may be using different color coding

between ligands and receptors as in Fig S8, however, not necessarily and leave this to the authors' decision.
Response: We appreciate Rev 2's suggestion of shifting the format of Fig. 6D to that of Fig S4A, which we have implemented.

5.Please double check the figure numbers throughout the text. I found some mismatches. In the section starting from P13 line 315, the figures seem to be S5 and not S4?
Response: Thanks for noticing this error; we double-checked all other fig citations.

Reviewer 3

First, in light of previously published studies, the title, summary statement, and abstract of the current manuscript should be revised. The title and summary statement imply that prospermatogonial development and spermatogonial stem cell genesis has not been profiled at the single cell level previously. However, this has already been accomplished by studies of Law et al., 2019, Nat Comm., which profiled fetal and neonatal prospermatogonia at more time points and with more total cells than the current study. The title and summary statement don't reflect novel advances that are made by the current study; in particular the studying of somatic cell populations in perinatal testes, which has not been reported previously, is left out completely. Also, Law et al. already described three different prospermatogonial subsets that emerge through developmental time, thus the authors statement in the abstract of the same discovery is redundant of existing knowledge and should be revised appropriately.
Response: We thank Rev 3 for these suggestions. In the revised MS, we changed our title to: 'Single-cell RNAseq analysis of testicular germ and somatic cell development during the perinatal period.' We also rewrote both the Summary Statement and Abstract so they better highlight our findings, including our analysis of testicular somatic cells, as suggested by Rev 3:
Revised Summary Statement: 'Testicular germ and somatic cells undergo major transitions during the perinatal period. Using single-cell RNAseq analysis, we molecularly chart these transitions and identify new cell subsets and stage-specific markers.'
Revised Abstract: 'Pro-spermatogonia (SG) serve as the gateway to spermatogenesis. Using single-cell RNA sequencing (RNAseq), we studied the development of ProSG, their SG descendants, and testicular somatic cells, during the perinatal period in mice. We identified both gene and protein markers for 3 temporally distinct ProSG cell subsets, including a migratory cell population with a distinct transcriptome from the previously defined T1- and T2-ProSG stages. This intermediate (I)-ProSG subset translocate from the center of seminiferous tubules to the spermatogonial stem cell (SSC) "niche" in its periphery soon after birth. We identified 3 undifferentiated SG subsets at postnatal day 7, each of which express distinct genes, including transcription factor and signaling genes. Two of these subsets have the characteristics of newly emergent SSCs. We also molecularly defined the development of Sertoli, Leydig, and peritubular myoid cells during the perinatal period, allowing us to identify candidate signaling pathways acting between somatic and germ cells in a stage-specific manner during the perinatal period. Our study provides a rich resource for those investigating testicular germ and somatic cell developmental during the perinatal period.'
Finally, we added a section in the Discussion on the topic of developing somatic cells (lines 443-480).

Previous studies that already profiled prospermatogonial and spermatogonial stem cell development still have not been appropriately represented in the introduction section. The authors' experiments largely duplicate the Law et al. study which is fine but the gaps in knowledge not filled by that study which are being addressed by the current study should be defined. Rather, the statement "recent scRNAseq studies have studied some of the events that preceed spermatogenesis, including SSC establishment" has been inserted to acknowledge Law et al. which is not really accurate because prospermatogonia were profiled throughout development time and at a greater number of development age points than the current study. It would be appropriate for the authors to add a few sentences that accurately reflect previous studies that have examined prospermatogonial development with scRNAseq and then define what the knowledge gaps are that their studies have addressed.
Response: We revised the Introduction to better highlight the study by Law et al.: 'Law et al. studied the transcriptome and cellular dynamics that accompany SSC specification through analysis of purified germ cells from multi-lineage reporter mice (Law et al., 2019). Using multiple

approaches, including scRNAseq analysis, they defined heterogenous ProSG and SG populations, along with the associated transcriptomes and putative regulatory networks. Germ-cell transplantation analysis revealed that Id4-eGFP+, but not Id4-eGFP-, E16.5 germ cells were able to rescue spermatogenesis in germ cell-deficient mice, suggesting that Id4-eGFP+ ProSG are fated to become SSCs.' (lines 58-64)

NOTE: While Rev 3 appears to be in support of publication of our MS, and thus it is presumably not important to justify the importance of our results, we still feel compelled to respectfully disagree with Rev 3's comments about the novelty of our findings.

'Law et al. already described three different prospermatogonial subsets that emerge through developmental time, thus the authors statement in the abstract of the same discovery is redundant of existing knowledge and should be revised appropriately…..The authors' experiments largely duplicate the Law et al. study which is fine but the gaps in knowledge not filled by that study which are being addressed by the current study should be defined.'
Response: We do not agree that our study is "redundant" and that our "experiments largely duplicate the Law et al. study." While we agree that Law et al. identified several pro-spermatogonia (ProSG) cell clusters, their main goal was to investigate the genesis of SSCs, not ProSG development. The following are features of our study that were not done by Law et al.: (i) we performed scRNAseq on unfractionated wild-type testicular cells, thereby avoiding the potential confounding effects of transgenes and FACS purification (both of which were features of the Law et al. study), (ii) we identified protein markers specifically labeling different ProSG and undifferentiated spermatogonia subsets, (iii) we defined transcription factors and transcription factor networks that are candidates to be involved in perinatal germ and somatic cell development and function, and (iv) we identified signaling pathways that are candidates to be involved in perinatal germ and somatic cell development and function. In addition, we identified a novel cell cluster—"I-ProSG"—that we show migrate from the seminiferous tubule center to the SSC niche in the periphery. We also showed that the other 2 ProSG clusters we identified have well-defined characteristics — one is proliferatively quiescent and the other is highly proliferative. Thus, the 3 ProSG clusters we identified largely mimic the ProSG developmental stages known to occur in vivo: proliferative quiescence, migration, and proliferative expansion. This, in turn, means that the datasets we provide in our MS for these 3 cell clusters (e.g., enriched genes, transcription factors, and signaling pathways) is a useful resource for the field. For example, the markers we identified that label these 3 ProSG stages can be used to functionally study these ProSG subsets, as well as SSCs establishment.

The authors' statement on lines 239-240 that SSCs emerge at P7 in mice needs to be reconsidered. If one is going define SSCs functionally based on the capacity to generate colonies of spermatogenesis, they emerge much earlier. Studies of Kubota et al., 2004, Biol. Reprod showed that cells in a P0 mouse testis can form spermatogenic colonies after transplant and Law et al., 2019, Nat Comm showed that some even exist in testes at E18.5.
Response: In our previous revision, we said the following: 'At P7, soon after SSCs first emerge (McLean et al., 2003; Shinohara et al., 2001), we identified two cell clusters—"SSC-1" and "SSC-2"—both of which express known undifferentiated SG and SSC-associated marker genes.' Thus, we did not claim that SSC emerge at P7. We had also previously cited the 2 papers referred to by Rev 3. Thus, we are puzzled as to the reviewer's concern. Is it that it is not clear whether SSCs continue to form at P7? We believe that the current evidence from the field does not answer this question. Unfortunately, because of strict space constraints, we do not have room to discuss this topic. Instead we have completely removed our previous statement about SSC emergence and the P7 time point, in case it leads to ambiguity.
With regard to the reviewer's comment that 'Law et al., 2019, Nat Comm showed that some even exist in testes at E18.5,' we agree that it is possible that SSCs exist even before birth. The evidence for this is Law et al's finding that purified E16.5 germ cells were able to rescue spermatogenesis, as measured by the germ-cell transplantation assay. This suggests that SSCs present at E16.5 conferred the rescue. However, an alternative interpretation is that there are no SSCs at E16.5 and that instead ProSG present at this time point undergo differentiation to form SSCs after they are transplanted. Transcriptome evidence that there are few (if any) SSCs at E16.5 comes from Law et al's scRNAseq data. They defined cluster 7 as the cluster harboring SSCs (from Figs. 4e-g in their paper), but careful analysis of this dataset shows that cluster 7 contains very few cells from E16.5 and P0 (1 and 9 cells, respectively). Instead, the vast majority of cluster 7 cells (1,665 cells) are

from P3 and P6, which are well-established time points when SSCs are present. Due to strict space constraints, we do not discuss this in the revised MS, but can do so if deemed important.

There is still lack of functional endpoints to support the authors' claims of identifying different prospermatogonial and SSC subsets. The data presented is descriptive and certainly suggestive but without a level of experimentation that assesses functional differences, the authors' conclusions are too strong and somewhat misleading to readers outside of the field. For example:
The abstract should be revised to indicate that the identified spermatogonial subsets are based on transcriptome signatures. As written, as reader of the abstract alone could be left with an impression that these subsets were defined based on functional differences.
Response: We revised the Abstract to say: 'Two of these subsets have the characteristics of newly emergent SSCs' (see complete Abstract above). Of note, we use the term "characteristics" rather than "transcriptome," as the evidence comes not only from transcriptome data but also the expression of SSC protein markers.
We also revised our description of SSC-related cell clusters in the Results and Discussion, as quoted in response to Rev 1.

Lines 586-592: the statement made about the most naïve nature of a spermatogonial subset needs to be clarified with wording that this is at the transcriptome level but functional assessment is lacking.
Response: We revised this section of the Discussion by first stating: 'How this transcriptome complexity is initially established is not known.' (line 420).
We then say: 'Using scRNAseq to analyze germ cells during the SSC establishment period, we identified two cell clusters of undifferentiated SG that express distinct patterns of genes, raising the possibility that SSCs are heterogenous even when first formed.' (lines 420-423), followed by 'While the functional significance of this difference in gene expression is not known, one possible....' (line 426-427).

In addition, the statement about a possible interconvertable nature of undifferentiated spermatogonial subsets needs to be qualified with wording that functional endpoints are lacking to support this.
Response: Below is how we have revised the relevant paragraph, which ends with a sentence about 'interconvertable states.' We believe it is clear from reading the entire paragraph that we are talking about cell clusters defined by scRNAseq analysis, not functionally defined SSC subsets:
'While the functional significance of this difference in gene expression is not known, one possible explanation is that SSC-s1 and SSC-s2 differ in their endurance as stem cells. Given that SSC-s1 is more primitive than SSC-s2, it may contain cells that tend to retain SSC activity for longer than SSC-s2 cells. Another possibility is that SSC-s1 and SSC-s2 are interconvertible states, in alignment with studies suggesting that mouse undifferentiated SG stages are in equilibrium (Hara et al., 2014).' (lines 426-431).

Moreover, stating that lineage tracing will be needed to ascertain developmental relationships does not necessarily imply that functional distinctions will be gained.
Response: We no longer mention lineage tracing.

Overall, the authors should better define what is meant by "SSC heterogeneity" as presented on line 580 and SSC states 1, s1, s2, and 2 as presented in Figures 2 and 7. If SSC is a functional definition of spermatogonia that support continual spermatogenesis or regenerate spermatogenesis after transplant, what is heterogeneous about this or how are different states defined? Heterogeneity in the expression level of certain genes may or may not correspond to a functional difference. In the absence of functional data, SSC subsets identified in the current study should be better described as being based on transcriptome heterogeneity. As presented, the connotation is functional differences.
Response: With regard to "SSC heterogeneity," we are uncertain what is being referred to, as we did not use that phrase in our previous revision. As described above, throughout the MS, we now better clarify that SSC-1, SSC-2, SSC-s1, and SSC-s2 are scRNAseq-defined cell clusters with distinct transcriptomes but with unknown functional differences.

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Third decision letter

MS ID#: DEVELOP/2019/183251

MS TITLE: Single-cell RNAseq analysis of testicular germ and somatic cell development during the perinatal period

AUTHORS: Kun Tan, Hye-Won Song, and Miles Wilkinson
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.