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Peer Review File

Pre-ciliated tubal epithelial cells are prone to initiation of high-grade serous ovarian carcinoma

Corresponding Author: Dr Alexander Nikitin

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This study by Flesken-Nikitin et al. utilized scRNA-seq to report a comprehensive census of cell types found in the mouse oviducts/fallopian tubes (uterine tubes is outdated and very confusing terminology). They sequenced the proximal and distal sections to investigate the characteristics underscoring the distal region's predisposition towards cancer initiation. In addition, they employed a combination of computational lineage trajectory projections (PHATE) and genetic cell fate. Their results detected a TE stem/progenitor cell population and that pre-ciliated cells may serve as a specific cell state susceptible to malignant transformation. My comments:

1. There is no conclusive evidence to support that Slc1a3+ cells are stem or progenitor cells. Cells labeled at 1DPI are also single or double and then at 30DPI, most cells are single or double with few clusters, even at 360 DPI, there is similar pattern. Not sure why 360DPI is not quantified. How many single labeled cells form their own clones? What was labeling efficiency and clone forming efficiency? This is a big claim without relevant data sets. From this data, it can be inferred that Slc1a3 are transitory secretory cells. Organoid data is showing no organoid formation in Slc1a3- cells, what about Pax8/Ovgp1+ Slc1a3-cells? These cells are known to give rise to organoids.
2. What is the expression pattern of keratin 5 in mouse tubes? How you know this is limited to preciliated cells? Why tumors in Krt5cre driven mice are not showing nuclear Pax8 expression and not histologically showing serous features? where are intermediate stages? STICs? Pax8cre and Ovgp1 data is expected and similar to previous reports.

3. In Results, Page 6, under section ‘Census of cell types of the mouse uterine tube’, Authors mentioned that the ‘16583 high quality cells were generated following sequencing and data processing of distal region samples’, how about proximal region samples? How many high-quality cells were obtained from proximal region because it is not mentioned in the results section text.

4. In Results, Figure 1B, Authors should also provide UMAP layout showing the cell types representing proximal and distal region SEPARATELY. This is a follow up from Figure 1A diagram of the uterine tube which Authors highlighted distal and proximal regions.

5. In Results, Figure 1B, Authors should provide a pie plot showing the proportions of these 18 cell type clusters in percentage so that we can see the difference between this cell types. Will be good to add the proximal data as well.

6. In Results, Figure 1C, Authors should provide Violin plots showing the expression of their selected epithelial markers (Epcam and Krt8), and secretory (Ovgp1) and ciliated (Foxj1) markers reflected in their results part Page 6. This violin plot will help to illustrate the proximal–distal patterning and marker gene expression.

7. In Results, Figure 2A, Authors should also mention in the results part, how many proximal epithelial subsets were detected. Supplementary Figure 3 has the proximal uterine data but not describe in the results section.

8. In Results, Figure 2, Authors should also perform GO terms/pathway analysis enriched in epithelial cell cluster between two regions proximal versus distal.

9. In Results, Figure 2B, Authors need sort of validation experiment using qPCR to validate marker of interest Slc1a3 in their samples.

10. In Results, Supplementary Figure 2F and 2G, it is not clear what ‘mD1, mD2 and mD4’ represents? Authors should at least clarify this abbreviation in the legend.

11. In Results, Figure 3A, it is not clear if this is differentiation trajectory among epithelial cells visualized through the PHATE dimensional reduction technique is focusing on the distal or proximal region?

12. In Results, Figure 3C and 3D need sort of quantification or plot analysis for the expression of Slc1a3 and Pax8 visualized over the PHATE embedding.

13. In Results, Page 7, last sentence, Authors mentioned that 'Pax8 expression is present in Slc1a3 positive cells but also extends towards early cilia-forming cells (presented in Figure 3d).' Does the Author have data containing negative control Slc1a3- cells to prove that Pax8 expression is also affected.

14. In Results, Figure 4B staining, the scale bar for the top and middle row is missing.

15. In Results, Figure 4E and 4F, is there any difference of FAM183B and OVGP1 expression specifically in proximal. It is difficult to appreciate because the value presented the y axis (%) maybe too small for cells expressing tdTomato in the proximal regions. In that case, Authors should provide a separate graph for this analysis.

16. In Results, Supplementary Figure 5a-d - Organoid formation by distal (a and b) and proximal (c and d). Authors should provide representative microscopic images of proximal and distal organoid culture at different days (example day 3, day 17 and day 28) of culture. Please provide a scale bar in all images. What happen to the organoid formation by proximal in Supplementary Figure 5C?

17. In Results, Supplementary Figure 5a-d, which day and passage was these organoid images taken? These organoids still need to be further validated using specific markers staining to confirm the features of distal and proximal tubal epithelium.

18. In addition, Authors should add the quantification of cell viability, organoid diameter and organoid size comparing between proximal and distal organoid formation.

19. In Results, Page 8 under section 'Slc1a3+ epithelial cells are not cancer-prone', there are labelling error in this whole section. All of this labelling (Figure 4a, 4b, 4c and 4d) needs to be rectify and change to 'Figure 5a, 5b, 5c and 5d'.

20. In Results, Page 9, under section 'Pre-ciliated cells are susceptible to malignant transformation', Authors mentioned that 'STICs (6 out of 12 cases) and/or HGSC (3 out of 12) were detected in 58% of mice by 400 days post induction (DPI).' However, I am unable to locate any data from figures reflecting this statement

21. In Figure 7C, the error bar is missing for the graph representing the frequency of neoplastic lesions (c).

22. In Discussion, Page 13, last paragraph, please correct 'In sum' to 'In summary..'

23. In In Methods, Page 15, under Experimental animals, it is unclear how many mice (n=?) were used in each experiments?

24. In Methods, Page 19, under TE organoid preparation, please correct 'ROCKi' to 'Y27632' consistent to how it is mentioned in Single-cell RNA sequencing library prep.

25. In Methods, Page 19, under FACS preparation and analysis, please add briefly the FACS parameters and software name used to analyze the FACS data.

Reviewer #2

(Remarks to the Author)
The authors have presented an RNA-seq atlas of proximal and distal uterine(fallopian) tube. They have gone on to show that a PAX8+ ciliated epithelial population is cancer prone.

In general a nice story.

The single cell analyses and figures should be improved.

1. There is a cluster in fig 1b labelled epithelial/fibroblast are these doublets? they are not mentioned in the manuscript.

2. What confidence do the authors have in the subpopulations of fibroblasts and stem-like epithelial cells? the markers in 1c don't really seem to separate the proposed sub-populations. The subclusters for fibroblasts are not even mentioned in the manuscript so it would be easy to just leave them as a higher level cluster.

3. Similar to point 1, Fig 2 has fibroblast-like cells in the epithelial subclustering. Are these doublets missed by doubletfinder?

4. Section "Slc1a3+ epithelial cells are not cancer-prone" mentions fig 4 but I think they mean fig 5.

5. Examining supp figure 2d, the epithelial/fibroblast and intermediate epithelial clusters appear to be doublets. Why aren't they removed from figure 1? Similarly the fibroblast-like cluster from figure 2 also appears to be doublets (supp figure 2e). It would make more sense to apply the doublet removal and then present the clusters AFTER doublet removal in figures 1 and 2.

Reviewer #3

(Remarks to the Author)
Review of “Pre-ciliated tubal epithelial cells are prone to initiation of high-grade serous ovarian carcinoma”

In this article Flesken-Nikitin et al., set out to investigate the cell states and lineage dynamics of epithelial cells in the mouse oviduct using a combination of single cell RNA sequencing and lineage tracing approaches. In addition, they aim to determine the susceptibility of specific cell types to malignant transformation. This is highly relevant as the human equivalent of the oviduct, the fallopian tube, is generally considered to be the tissue of origin of high-grade serous ovarian carcinoma and very little is currently know about the lineage hierarchy. The authors initially set out to characterise the cells in the mouse oviduct by performing single cell RNA sequencing and were successfully able to identify many cell types based on their transcriptomes and predict lineage hierarchies using trajectory and pseudo time analysis. From this they identified a potential stem cell population marked by Slc1a3 which they went on to show had progenitor characteristics using lineage tracing. Surprisingly, they found introduction of genetic perturbations seen in HGSOC patients in these cells did not induce transformation but resulted in apoptosis. Interestingly, when the authors targeted another epithelial cell population, which they suggest is transitioning to becoming multiciliated, they see enhanced STIC and HGSOC formation. This result is important as it goes against the current dogma that PAX8+ progenitors are the cell of origin of HGSOC and raises questions about intrinsic determents of malignant susceptibility. These results are supported by a previous publication using human data, which suggested an intermediate cell state as the cell of origin marked by RUNX3 (https://pubmed-ncbi-nlm-nih-gov.ezp.lib.cam.ac.uk/33852846/).

Overall, the experimental approach is well thought through and the findings potentially important in the field of ovarian cancer and our understanding of susceptibility and cancer development. However, the authors have not shown significant validation of their scRNAseq to fully support their claims which limits the impact of their work. Below I have detailed my specific concerns and suggestions.

Major:

• The authors have not considered the impact of the oestrus cycle in their dataset. It has been previously shown that the transcriptomes of cells in the mouse oviduct change at different stages of the oestrus cycle in response to hormonal changes (https://pubmed.ncbi.nlm.nih.gov/33818810/). Because the authors have combined many mice, which were not staged, it is impossible from the scRNAseq data alone to know if distinct clusters represent distinct cell types/states or the same cell types at different stages of the oestrus cycle. Validation of their clusters by other methods, such as immunofluorescence at different oestrus cycle stages, would be important to help interpret their findings.

• It is currently believed that the mouse oviduct contains PAX8+ secretory and PAX8- multiciliated cells, with some evidence that all epithelial cells are PAX8+ in proximal regions (https://pubmed.ncbi.nlm.nih.gov/34496237/). Considering this, the results in “Characterization of distal epithelial cell states” that report PAX8- secretory cells in all regions is surprising. It would be important for the authors to either validate their findings or consider and discuss the limitations of scRNAseq which is prone to high dropout rates. It is possible PAX8- cells in their dataset do express low levels of PAX8 which is not detected by this method.

• The FACS data in S fig5 is hard to interpret and does not provide good evidence that Slc2a3 marks stem/progenitors. The authors show that Tdt/Slc2a3+ cells have a higher organoid forming capacity compared to Tdt- cells. As the majority of Tdt-cells will be stromal this is not surprising. A more refined experiment would compare the organoid forming capacity of Slc2a3 expressing and non-expressing cells in just the non-ciliated epithelial population.

• It is suggested that Trp53 inactivation leads to elimination of Slc1a3+ cells. A control experiment which shows elimination of Trp53- Slc1a3+ cells would be important to confirm that Rb1 loss is not also required with Trp53 loss to induce apoptosis.

• The authors claim in figure 7 that transitional pre-ciliated Krt5+ cells are highly susceptible to transformation. In support of their claims that Krt5/Prom1+ cells are transitional, there is a recent lineage tracing study which also suggests Prom1+ cells are transitional (https://www-ncbi-nlm-nih-gov.ezp.lib.cam.ac.uk/pmc/articles/PMC10508696/). In the current manuscript however, more direct evidence is required to show Krt5+ cells are transitional. Using their Krt5-CreERT lineage tracing system the authors could show that labelled cells undergo differentiation thereby validating their scRNAseq analysis.

Minor:

• The authors have used the term “uterine tube” throughout the article. I would recommend using oviduct instead as this is the most widely used annotation.

• In the introduction the authors state that STICs are found exclusively in the distal portion of the fallopian tube. While current evidence suggest STICs are more likely to be found in the distal region I do not think we can currently say they are exclusively found here. One factor which hinders our current knowledge of this is the use of screening methods such as SEE-FIM, which focus on the distal region.

• The authors mention STICs can be formed in mouse models targeting Trp53, Brca1, Brca2 and Rb1 in PAX8+ cells. In the corresponding reference however, the research team targeted Pten and not Rb1. Supporting Rb1 loss is another study targeting OVGP1 expressing cells (https://www-ncbi-nlm-nih-gov.ezp.lib.cam.ac.uk/pmc/articles/PMC5568969/).

• In S Fig 2 : it is unclear what locations mD1/2/4 mean.

• Tamoxifen is an estrogen agonist and has previously been shown to impact the homeostasis and development of the oviduct (https://pubmed.ncbi.nlm.nih.gov/9364013/)(https://pubmed.ncbi.nlm.nih.gov/9685853/). Its use in the Slc1a3 lineage tracing experiment needs to be considered and potential side effects acknowledged. A control experiment to compare the pattern of Slc1a3 immunofluorescent staining with Tdtomato to determine if the administration of tamoxifen has any impact on the expression pattern of Slc1a3 would be informative.

• The authors mention in their model that STICs are forming near the TE-mesothelial junctions. Can they provide evidence of this.

• Could the authors give some more information on the HGSOC neoplasms that are forming in their Krt5-CreERT model. Do they form in the oviduct? On the ovary? Is there metastatic spread to the peritoneal cavity?

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have largely addressed my comments.

For the 3 fibroblast populations shown in Figure 1, it would have been useful to at least mention them in the main text and highlight marker genes that were differentially expressed between them.

Similarly for the two Stem-epithelial in figure 1, it would have been useful to mention the markers that separate them. (e.g. are Slc1a3 and Cepd, differentially expressed between stem-epithelial 1 and stem-epithelial 2?).

It would also be useful to state which cells from figure 1 were taken forward into figure 2.

I think the authors have done a good job at highlighting which populations are likely to be doublets. The inclusion of the fibroblast-like population in Fig2 doesn't really add anything to the story, but they have included the caveat that it is likely a doublet.

Reviewer #3

(Remarks to the Author)
I would like to thank the authors for addressing all my comments. I am satisfied with their responses and the additional experiments they have included.

I have one minor query concerning the origin of the peritoneal metastatic tumours produced in their Krt5 model in figure 7. There is an assumption that these originate from the premalignant lesions in the oviduct. Have the authors checked if their Creline is specific to the oviduct epithelium and does not hit cells in the ovarian surface epithelium, which could be an alternative source of these tumours. An experiment to show that their Krt5 model does not hit the ovarian surface epithelium and/or comparative genomic sequencing of the metastatic tumours with lesions in the oviduct from the same mouse would confirm their origin in the oviduct.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
I would like to thank the authors for addressing all my comments and I agree that their findings supporting a "transient amplifying "hypothesis for Krt5+ cells.

I suggest that a schematic summarizing the authors findings/thoughts on the epithelial cell hierarchy in the oviduct, including relevant markers would be helpful. Also a statement outlining the limitations of their findings.

I do not think anymore experiments are required and would like to congratulate the authors for their excellent work.

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Point-by-point response to the reviewers' comments

Reviewer #1, expertise in lineage tracing and ovarian cancer (Remarks to the Author):

This study by Flesken-Nikitin et al. utilized scRNA-seq to report a comprehensive census of cell types found in the mouse oviducts/fallopian tubes (uterine tubes is outdated and very confusing terminology). They sequenced the proximal and distal sections to investigate the characteristics underscoring the distal region's predisposition towards cancer initiation. In addition, they employed a combination of computational lineage trajectory projections (PHATE) and genetic cell fate. Their results detected a TE stem/progenitor cell population and that pre-ciliated cells may serve as a specific cell state susceptible to malignant transformation.

We appreciate very thorough and helpful critiques by Reviewer #1.

We adhere to a view that a unifying standardized terminology greatly benefits comparative studies involving animals to understand human disease. As exemplified by comments by Reviewer #1 (beginning of remarks) and Reviewer #3 (comment 6), both Fallopian tube and oviduct are commonly used by different groups of investigators. This indeed can be confusing. It is our opinion that "uterine tube is the most anatomically accurate name for this structure. The term "uterine tube" precisely describes its location and function as a tube leading from the ovary to the uterus. "Fallopian tube," named after the 16th-century Italian anatomist Gabriele Falloppio, is a more traditional term, but it's less descriptive anatomically. "Oviduct" is also used and is anatomically descriptive, indicating a duct for the ovum (egg), but it is less specific to human anatomy and is often used in the context of various animals. We use the "uterine tube" as the most unifying term allowing to be efficiently used for comparative human/mouse studies typical in experimental research (see for example, Flesken-Nikitin et al., *Nature* 495: 183–184, 2013, doi:10.1038/nature11962, and Fu et al, *Dis. Model. Mech*. 13(10):dmm047035. doi: 10.1242/dmm.047035). Hopefully our current manuscript will further facilitate more broad acceptance of the term "uterine tube" by research community using both species in their research. We have included our reasons for using this term in the revised Methods in section "Anatomical nomenclature".

My comments:

1.1. *There is no conclusive evidence to support that Slc1a3+ cells are stem or progenitor cells. Cells labeled at 1DPI are also single or double and then at 30DPI, most cells are single or double with few clusters, even at 360 DPI, there is similar pattern.*

We use a definition of stem cells as cells able to reproduce themselves and produce more differentiated progeny. Frequently, it is very difficult to establish if newly identified cell populations represent "pure" stem cells or also contain some of their immediate progeny progressing towards differentiation. In this case we use a term "stem/progenitor" cells. To meet these definitions, we use a combination of computational lineage prediction, lineage tracing and organoid formation. To the best of our knowledge, a combination of these methods offers a rigorous support to our conclusion that Slc1a3+ cells are stem/progenitor cells.

Adult tubal epithelium proliferates slowly, and some cells are expected to remain single or in small groups. However, the overall increase in the number of tdTomato+ cells is very clear by 360 days (Fig. 3b-c). To facilitate the quantification of tdTomato+ cluster formation we have performed labeling of Slc1a3-CreER+ Ai9 cells with lower dose of tamoxifen (10 μg/bw). At this dose, we

have not observed any clusters at 1 DPI and could quantify clones by 30 DPI (Supplementary Figure 8).

1.2. Not sure why 360DPI is not quantified.

We have added quantification of 360 DPI point (Figure 3, c-f).

1.3. How many single labeled cells form their own clones? What was labeling efficiency and clone forming efficiency?

As discussed in 1.1, we estimated clone formation in experiments with low dose of tamoxifen (10 µg/bw). At that dose, about 1.3% of cells are labeled at 1 DPI (Supplementary Figure 8c). This number increases to 2.3% by 30 DPI. No clusters are observed at 1 DPI, but about 26% of tdTomato+ cells are in clusters by 30 DPI (Figure 8d).

1.4. This is a big claim without relevant data sets. From this data, it can be inferred that Slc1a3 are transitory secretory cells.

A possibility of Slc1a3+ cells being transitory secretory cells is unlikely for several reasons: (1) Our single cell transcriptome lineage trajectory prediction shows clear bifurcation of cells towards secretory and ciliated cell lineages with Slc1a3 cells being at the origin of this bifurcation (Figures 2 and 6). (2) Slca1a3+ cells contribute to formation of both ciliated and secretory cells in cell lineage tracing experiments (Figure 3). Furthermore, our additional organoid experiments show that organoids formed by Slc1a3+ cells have higher propensity for formation of ciliated cells, as compared to those formed by Slc1a3- cells (Figure 4). Secretory transitory cells are expected to contribute to more mature secretory cells not ciliated cells. (3) Slc1a3+ cells live at least 360 days (Figure 3). Transitory cells are not expected to be long living. Thus, we do not see how it can be inferred that Slc1a3 are transitory secretory cells based on our data.

1.5. Organoid data is showing no organoid formation in Slc1a3- cells, what about Pax8/Ovgp1+ Slc1a3-cells? These cells are known to give rise to organoids.

To address the capacity of SLC1A3+/- cells to give rise to organoids, we completed additional MACS experiments to isolate SLC1A3+ epithelial cells from pure epithelial cell populations. Our additional studies show organoid formation by Slc1a3- cells but at lower frequency (Figure 4). Both SLC1A3+ and SLC1A3- cell derived organoids express secretory marker OVGP1 (Figure 4a and b). However, SLC1A3+ epithelial cells give rise to organoids with significantly more ciliation. According to our results, cells expressing *Pax8* co-express *Slc1a3* except for pre-ciliated *Krt5*+ cells, which suggests that Slc1a3 may be a subset of *Pax8*+ cells with organoid-forming capabilities (Figure. 6). We are not aware of any studies describing TE organoid formation by Pax8/Ovgp1+ Slc1a3-cells.

2.1. What is the expression pattern of keratin 5 in mouse tubes?

According to our single cell transcriptome date, *Krt5* is expressed only in the distal tubal epithelium (Figure 6). We have supplied lineage tracing experiments to better characterize *Krt5*+ pre-ciliated cells (Figure 7).

2.2. How you know this is limited to preciliated cells?

Krt5 expression within the single-cell data is limited to the pre-ciliated cells (Figure 6f). We also included staining and quantification of tdTomato+ cells from *Krt5*-Cre Ai9 mice with FOXJ1,TRP73, and OVGP1 to characterize this population (Figure 7).

2.3. Why tumors in Krt5cre driven mice are not showing nuclear Pax8 expression and not histologically showing serous features?

We have added additional examples of neoplastic lesions forming in our model (Figure 7c-f). In our experience, HGSC cells may have both nuclear and cytoplasmic PAX8 staining. To further characterize our material, we have also added stainings for other diagnostic markers of HGSC, such as Wilms tumor 1, P16 and Ki67 (Figure 7c-f). Solid pattern of growth is common for HGSC, in addition to slit-like spaces, papillary and glandular patterns. Pathological studies of our mouse models were performed by pathologists Dr. Fu, Dr. Ellenson and Dr. Nikitin. All of them have experience in comparative pathological evaluation of human and mouse neoplasms of the female reproductive tract.

2.4. Where are intermediate stages? STICs?

We have included additional images of both early and advanced neoplastic lesions to better show different stages (Figure 7c-f and Supplementary Figure 11).

2.5. *Pax8cre and Ovgp1 data is expected and similar to previous reports.*
We are not sure what does Reviewer #1 requests. Characterization of Pax8-Cre model has been reported previously (Perets et al. 2013, Fu et al., 2020). It is also present in our manuscript (Figure 6a and b, and Supplementary Table 4). According to our lineage tracing experiments, expression of Ovgp1-Cre transgene labels a large fraction of distal ciliated TE cells already at 1 DPI. We expect to address this topic in another manuscript.

3. In Results, Page 6, under section 'Census of cell types of the mouse uterine tube', Authors mentioned that the '16583 high quality cells were generated following sequencing and data processing of distal region samples', how about proximal region samples? How many high-quality cells were obtained from proximal region because it is not mentioned in the results section text.

We demonstrate the design of the scRNA-seq data collected from proximal and distal sections within Supplementary Figure 1. In depth description of the proximal region exceeds the scope of our manuscript. However, we would like to include the already collected proximal data for data availability purposes,.These data will be given more attention in a future manuscript focusing on comparative investigation of the distal and proximal regions of the uterine tube.

4. In Results, Figure 1B, Authors should also provide UMAP layout showing the cell types representing proximal and distal region SEPARATELY. This is a follow up from Figure 1A diagram of the uterine tube which Authors highlighted distal and proximal regions.

We have stated in Figure legends that Figures 1, 2, 6 and Supplementary Figure 2 address the distal datasets and the proximal data is shown within Supplementary Fig 4.

5. *In Results, Figure 1B, Authors should provide a pie plot showing the proportions of these 18 cell type clusters in percentage so that we can see the difference between this cell types. Will be good to add the proximal data as well.*

We included tile mosaic visualizations for the proportions of the identified clusters. These plots can be seen in Supplementary Figure 2 and Supplemental Figure 3 for distal and proximal datasets respectively.

6. In Results, Figure 1C, Authors should provide Violin plots showing the expression of their selected epithelial markers (Epcam and Krt8), and secretory (Ovgp1) and ciliated (Foxj1) markers reflected in their results part Page 6. This violin plot will help to illustrate the proximal–distal patterning and marker gene expression.

We included these plots for both the main datasets and the epithelial subsets for both the distal and proximal regions (Supplementary Figure 2 and Supplemental Figure 4).

7. *In Results, Figure 2A, Authors should also mention in the results part, how many proximal epithelial subsets were detected. Supplementary Figure 3 has the proximal uterine data but not describe in the results section.*

We do demonstrate this within Supplementary Figure 1 but have made this clearer within the written text. We have also added this information into the results section (page 7).

8. *In Results, Figure 2, Authors should also perform GO terms/pathway analysis enriched in epithelial cell cluster between two regions proximal versus distal.*

This analysis is outside the scope of this manuscript and will better fit with a separate manuscript addressing comparative aspects of distal and proximal regions.

9. *In Results, Figure 2B, Authors need sort of validation experiment using qPCR to validate marker of interest Slc1a3 in their samples.*

We have performed immunostaining for SLC1A3+ cells to validate our single cell transcriptome and lineage tracing data (Figure 3b).

10. *In Results, Supplementary Figure 2F and 2G, it is not clear what 'mD1, mD2 and mD4' represents? Authors should at least clarify this abbreviation in the legend.*

We have made this clearer within the figure legends and we have included this within the sample preparation graphic (Supplementary Figure 1).

11. *In Results, Figure 3A, it is not clear if this is differentiation trajectory among epithelial cells visualized through the PHATE dimensional reduction technique is focusing on the distal or proximal region?*

We revised the figure legends to make it clearer that the dataset is only distal TE cells (Figure 2 legend of the revised manuscript).

12. *In Results, Figure 3C and 3D need sort of quantification or plot analysis for the expression of Slc1a3 and Pax8 visualized over the PHATE embedding.*

Quantification of the binned PHATE figure is demonstrated within Figure 6d. This figure shows the z-score expression of key genes that were predicted to play a role in epithelial cell differentiation.

13. *In Results, Page 7, last sentence, Authors mentioned that 'Pax8 expression is present in Slc1a3 positive cells but also extends towards early cilia-forming cells (presented in Figure 3d).' Does the Author have data containing negative control Slc1a3- cells to prove that Pax8 expression is also affected.*

These data are best shown in violin plots of Figure 6e, where there is a Pax8+/Slc1a3- group of cells detected. Analysis of mutual impacts of Slc1a3 and Pax8 on their expression is beyond the scope of this study. This is an interesting question and will be addressed in our future mechanistic studies.

14. *In Results, Figure 4B staining, the scale bar for the top and middle row is missing.*

To maximize the amount of visible morphological data we routinely use a single scale bar per plate. Please see our previous publications (e.g., Flesken-Nikitin et al, *Nature* 495: 183–184, 2013, doi:10.1038/nature11962, Fu et al., *Annu. Rev. Pathol*. 13:71-92, 2018, doi: 10.1146/annurev-pathol-020117-043935., Fu et al., *Nat. Commun*. 11:84, 2020, doi.org/10.1038/s41467-019-13847-2., 2018). The relevant scale of each image is explained within the legend.

15. *In Results, Figure 4E and 4F, is there any difference of FAM183B and OVGP1 expression specifically in proximal. It is difficult to appreciate because the value presented the y axis (%) maybe too small for cells expressing tdTomato in the proximal regions. In that case, Authors should provide a separate graph for this analysis.*

The scale and the counts of the proximal quantification demonstrate that these cells exist more within the distal uterine tube (Figure 3e and 3f). While the proximal portion is of importance, the scope of this manuscript focuses on the cancer-prone cell states within the distal TE. In depth analysis of the proximal data will be provided in another manuscript.

16. *In Results, Supplementary Figure 5a-d - Organoid formation by distal (a and b) and proximal (c and d). Authors should provide representative microscopic images of proximal and distal organoid culture at different days (example day 3, day 17 and day 28) of culture. Please provide a scale bar in all images. What happen to the organoid formation by proximal in Supplementary Figure 5C?*

We have addressed these concerns by additionally performing MACS experiments to isolate SLC1A3+ epithelial cells (Figure 4). We isolated these cells only from the distal portion of the uterine tube, and isolated for pure epithelial populations. We grew organoids for up to 14 days and characterized them histologically. We have also addressed the quality of the analysis within the Supplementary Figure 9. The proximal data will be provided in another manuscript.

17. *In Results, Supplementary Figure 5a-d, which day and passage was these organoid images taken? These organoids still need to be further validated using specific markers staining to confirm the features of distal and proximal tubal epithelium.*

All organoids are directly isolated from uterine tubes within these studies. We have also included staining validation of TE markers and quantification of cilia (Figure.4). The proximal data will be provided in another manuscript addressing comparative aspects of distal and proximal regions.

18. In addition, Authors should add the quantification of cell viability, organoid diameter and organoid size comparing between proximal and distal organoid formation.

We have built upon these organoid experiments, and we quantified data that are relevant to our conclusions (Figure 4 and Supplementary Figure 9). The proximal data will be provided in another manuscript addressing comparative aspects of distal and proximal regions.

19. *In Results, Page 8 under section 'Slc1a3+ epithelial cells are not cancer-prone', there are labelling error in this whole section. All of this labelling (Figure 4a, 4b, 4c and 4d) needs to be rectify and change to 'Figure 5a, 5b, 5c and 5d'.*

We apologize for this mislabeling. All labeling has been corrected.

20. *In Results, Page 9, under section 'Pre-ciliated cells are susceptible to malignant transformation', Authors mentioned that 'STICs (6 out of 12 cases) and/or HGSC (3 out of 12) were detected in 58% of mice by 400 days post induction (DPI).' However, I am unable to locate any data from figures reflecting this statement.*

This information is provided in the Supplementary Table 4. Examples of early and advanced lesions are shown in Figure 6 a, b, Figure 7 and Supplementary Figure 11.

21. *In Figure 7C, the error bar is missing for the graph representing the frequency of neoplastic lesions (c).*

This figure has been removed because it is redundant with information provided in the Supplementary Table 4.

22. *In Discussion, Page 13, last paragraph, please correct 'In sum' to 'In summary.*

Corrected.

23. *In In Methods, Page 15, under Experimental animals, it is unclear how many mice (n=?) were used in each experiments?*

All numbers of mice are indicated in the legends to figures and Supplementary Tables 2 and 4.

24. *In Methods, Page 19, under TE organoid preparation, please correct 'ROCKi' to 'Y27632' consistent to how it is mentioned in Single-cell RNA sequencing library prep.*

Corrected.

25. *In Methods, Page 19, under FACS preparation and analysis, please add briefly the FACS parameters and software name used to analyze the FACS data.*

This has been added to the Methods section.

Reviewer #2, expertise in ovarian cancer and scRNAseq (Remarks to the Author):
The authors have presented an RNA-seq atlas of proximal and distal uterine(fallopian) tube. They have gone on to show that a PAX8+ ciliated epithelial population is cancer prone. In general a nice story. The single cell analyses and figures should be improved.

We thank the reviewer #2 for a positive assessment of our work,

1. *There is a cluster in fig 1b labelled epithelial/fibroblast are these doublets? they are not mentioned in the manuscript.*

We suspect that this population is a doublet based on our DoubletFinder results (Supplementary Figure 2d). However, since this population is excluded from our analysis of the epithelial populations, we left it to maintain the reproducibility of our dataset for others to recreate the object. We used discretion for doublet removal, since DoubletFinder may eliminate transitional cell states in which we strive to capture to fully characterize epithelial cell lineages.

2. *What confidence do the authors have in the subpopulations of fibroblasts and stem-like epithelial cells? the markers in 1c don't really seem to separate the proposed sub-populations. The subclusters for fibroblasts are not even mentioned in the manuscript so it would be easy to just leave them as a higher level cluster.*

Pax8/*Itga6*/*Cd44* are previously identified markers of cells within the uterine tube known to have stem-like properties. *Foxj1* and *Ovgp1* further split secretory and ciliated cell populations. Although we do not investigate the fibroblast populations, we leave them as separate clusters so that we do not manipulate our scRNA-seq results to encourage reproducibility. Further investigation into the fibroblasts is a topic for future study.

3. *Similar to point 1, Fig 2 has fibroblast-like cells in the epithelial subclustering. Are these doublets missed by doubletfinder?*

To justify that this population is a doublet, we have included Supplementary Figure 3, which shows that this population shares both fibroblast and epithelial markers. To remain consistent with our previous dataset construction of the entire distal uterine tube, we left the possible doublets within our characterization plots. However, we also acknowledge that this population is most likely a doublet within the text (page 7) to justify its removal for the trajectory analysis performed in Figure 6.

4. *Section "Slc1a3+ epithelial cells are not cancer-prone" mentions fig 4 but I think they mean fig 5.*

We apologize for this mislabeling. This has been corrected in the revised manuscript.

5. *Examining supp figure 2d, the epithelial/fibroblast and intermediate epithelial clusters appear to be doublets. Why aren't they removed from figure 1? Similarly the fibroblast-like cluster from figure 2 also appears to be doublets (supp figure 2e). It would make more sense to apply the doublet removal and then present the clusters AFTER doublet removal in figures 1 and 2.*

The reasoning for keeping the intermediate epithelial population is that we do not know much about the epithelial lineage dynamics, which may be confounded by it having transitional states. To remain consistent among our analyses, we do not remove any clusters as a result of their DoubletFinder scores.

While we do not remove the ‘Fibroblast-like’ population for the characterization of the epithelial subset, we demonstrate that this cluster is most likely a doublet per Supplementary Figure 3, and we remove this population for our pseudotime binning analysis. In contrast, other scRNA-seq studies of human uterine tubes report epithelial cells with “EMT markers” such as those identified in our ‘Fibroblast-like’ population (https://pubmed.ncbi.nlm.nih.gov/32049047/, https://pubmed.ncbi.nlm.nih.gov/33852846/, https://pubmed.ncbi.nlm.nih.gov/35320732/). For

our dataset to serve as a bridge between mouse and human sequencing studies, we claim to suspect this population as a doublet, but we continue to characterize it aside from our pseudotime binned trajectory analysis.

Reviewer #3, expertise in lineage tracing, oviduct and organoids (Remarks to the Author):
Review of "Pre-ciliated tubal epithelial cells are prone to initiation of high-grade serous ovarian carcinoma" In this article Flesken-Nikitin et al., set out to investigate the cell states and lineage dynamics of epithelial cells in the mouse oviduct using a combination of single cell RNA sequencing and lineage tracing approaches. In addition, they aim to determine the susceptibility of specific cell types to malignant transformation. This is highly relevant as the human equivalent of the oviduct, the fallopian tube, is generally considered to be the tissue of origin of high-grade serous ovarian carcinoma and very little is currently know about the lineage hierarchy. The authors initially set out to characterise the cells in the mouse oviduct by performing single cell RNA sequencing and were successfully able to identify many cell types based on their transcriptomes and predict lineage hierarchies using trajectory and pseudo time analysis. From this they identified a potential stem cell population marked by Slc1a3 which they went on to show had progenitor characteristics using lineage tracing. Surprisingly, they found introduction of genetic perturbations seen in HGSOC patients in these cells did not induce transformation but resulted in apoptosis. Interestingly, when the authors targeted another epithelial cell population, which they suggest is transitioning to becoming multiciliated, they see enhanced STIC and HGSOC formation. This result is important as it goes against the current dogma that PAX8+ progenitors are the cell of origin of HGSOC and raises questions about intrinsic determents of malignant susceptibility. These results are supported by a previous publication using human data, which suggested an intermediate cell state as the cell of origin marked by RUNX3 (https://pubmed-ncbi-nlm-nih-gov.ezp.lib.cam.ac.uk/33852846/). Overall, the experimental approach is well thought through and the findings potentially important in the field of ovarian cancer and our understanding of susceptibility and cancer development. However, the authors have not shown significant validation of their scRNAseq to fully support their claims which limits the impact of their work. Below I have detailed my specific concerns and suggestions.

We appreciate very thorough comments and an encouraging assessment of our work by Reviewer #3.

Major:
1 • *The authors have not considered the impact of the oestrus cycle in their dataset. It has been previously shown that the transcriptomes of cells in the mouse oviduct change at different stages of the oestrus cycle in response to hormonal changes (https://pubmed.ncbi.nlm.nih.gov/33818810/). Because the authors have combined many mice, which were not staged, it is impossible from the scRNAseq data alone to know if distinct clusters represent distinct cell types/states or the same cell types at different stages of the oestrus cycle. Validation of their clusters by other methods, such as immunofluorescence at different oestrus cycle stages, would be important to help interpret their findings.*

All materials for single cell transcriptome studies were collected in the estrus stage of the estrous cycle. To clarify this point we have improved respective phrasing in the Single-cell RNA sequencing library preparation section of Methods.

2. • *It is currently believed that the mouse oviduct contains PAX8+ secretory and PAX8- multiciliated cells, with some evidence that all epithelial cells are PAX8+ in proximal regions (https://pubmed.ncbi.nlm.nih.gov/34496237/). Considering this, the results in "Characterization of distal epithelial cell states" that report PAX8- secretory cells in all regions is surprising. It would*

be important for the authors to either validate their findings or consider and discuss the limitations of scRNAseq which is prone to high dropout rates. It is possible PAX8- cells in their dataset do express low levels of PAX8 which is not detected by this method.

We agree that interpretation of single cell transcriptome analysis should be confirmed by other methods, such as immunostaining. Thus, we investigated co-localization of PAX8 and secretory markers OVGP1 and UPK1A by immunostaining (Supplementary Figure 7). In agreement with transcriptome data our results show that some cells expressing secretory markers have very minute if any amounts of PAX8. We also acknowledge that dropouts are a limitation of scRNA-seq and have discussed this possibility within the discussion section (page 8).

3. • *The FACS data in S fig5 is hard to interpret and does not provide good evidence that Slc2a3 marks stem/progenitors. The authors show that Tdt/Slc2a3+ cells have a higher organoid forming capacity compared to Tdt- cells. As the majority of Tdt- cells will be stromal this is not surprising. A more refined experiment would compare the organoid forming capacity of Slc2a3 expressing and non-expressing cells in just the non-ciliated epithelial population.*

We thank the Reviewer #3 for this great suggestion. We completed MACS experiments to isolate SLC1A3+ epithelial cells from pure epithelial cell populations. These studies confirmed preferential organoid formation by SLC1A3+ cells. Furthermore, SLC1A3+ epithelial cells gave rise to organoids with significantly more ciliation than SLC1A3- epithelial cells. We have added additional data for the MACS method (Figure 4 and Supplementary Figure 9), to augment the FACS data.

4. • *It is suggested that Trp53 inactivation leads to elimination of Slc1a3+ cells. A control experiment which shows elimination of Trp53- Slc1a3+ cells would be important to confirm that Rb1 loss is not also required with Trp53 loss to induce apoptosis.*

We have performed the requested experiment and have found no evidence that *Rb1* loss is required for elimination of Slc1a3 cells. This is consistent with our earlier observations of *Rb1* but not *Trp53* loss in cells originating from Slc1a3+ cells (revised Figure 5).

5.• *The authors claim in figure 7 that transitional pre-ciliated Krt5+ cells are highly susceptible to transformation. In support of their claims that Krt5/Prom1+ cells are transitional, there is a recent lineage tracing study which also suggests Prom1+ cells are transitional (https://www-ncbi-nlm-nih-gov.ezp.lib.cam.ac.uk/pmc/articles/PMC10508696/). In the current manuscript however, more direct evidence is required to show Krt5+ cells are transitional. Using their Krt5- CreERT lineage tracing system the authors could show that labelled cells undergo differentiation thereby validating their scRNAseq analysis.*

We have added the requested experiments and demonstrate that Krt5+ cells increasingly co-express markers of ciliogenesis (FOXJ1 and TRP53) but not secretory marker OVGP1 (Figure 7a and b).

Minor: •

6. *The authors have used the term "uterine tube" throughout the article. I would recommend using oviduct instead as this is the most widely used annotation.*

Please see our response to Reviewer #1. That Reviewer is favoring using "Fallopian tube". In our view, the "uterine tube" is the most appropriate and unifying term for comparative studies of humans and mice.

7.• *In the introduction the authors state that STICs are found exclusively in the distal portion of the fallopian tube. While current evidence suggest STICs are more likely to be found in the distal region I do not think we can currently say they are exclusively found here. One factor which hinders our current knowledge of this is the use of screening methods such as SEE-FIM, which focus on the distal region.*

In collaboration with other pathologists, we preformed excessive evaluation of tubal lesions in both distal and proximal regions of the human uterine tube (Schmoeckel et al., *Modern Pathology* 30:1241-1250, 2017, doi: 10.1038/modpathol.2017.53). Those studies have found presence of SCOUTs but not STICs in the proximal region.

8. • *The authors mention STICs can be formed in mouse models targeting Trp53, Brca1, Brca2 and Rb1 in PAX8+ cells. In the corresponding reference however, the research team targeted Pten and not Rb1. Supporting Rb1 loss is another study targeting OVGP1 expressing cells (https://www-ncbi-nlm-nih-gov.ezp.lib.cam.ac.uk/pmc/articles/PMC5568969/).*

We thank the Reviewer #3 for this advise. We have added Pten and indicated an additional manuscript describing targeting Rb1 in Pax8+ cells. (Fu et al., Dis. Model. Mech. 13(10):dmm047035. doi: 10.1242/dmm.047035).

9.• *In S Fig 2 : it is unclear what locations mD1/2/4 mean.*

We have clarified this within Supplementary Figure 1 and within the figure caption for Supplementary Figure 2.

10.• *Tamoxifen is an estrogen agonist and has previously been shown to impact the homeostasis and development of the oviduct (https://pubmed.ncbi.nlm.nih.gov/9364013/)(https://pubmed.ncbi.nlm.nih.gov/9685853/). Its use in the Slc1a3 lineage tracing experiment needs to be considered and potential side effects acknowledged. A control experiment to compare the pattern of Slc1a3 immunofluorescent staining with Tdtomato to determine if the administration of tamoxifen has any impact on the expression pattern of Slc1a3 would be informative.*

In our model system, it is impossible to induce and evaluate tdTomato expression without tamoxifen administration. However, to address a possible impact of Tamoxifen on SLC1A3+ cells we evaluated their frequency 1 and 30 days after tamoxifen administration. No significant changes were observed (Supplementary Figure 2). This is consistent with recent studies showing no significant impact of estrous cycle on gene expression in the tubal epithelium (Winkler et al., *Cell* 187, 1–18, 2024, https://doi.org/10.1016/j.cell.2024.01.021)

11. • *The authors mention in their model that STICs are forming near the TE-mesothelial junctions. Can they provide evidence of this.*

Examples of TE-mesothelial junction location of early dysplastic lesions are shown in Figure 7c and Supplementary Figure 11a.

12 • *Could the authors give some more information on the HGSOC neoplasms that are forming in their Krt5-CreERT model. Do they form in the oviduct? On the ovary? Is there metastatic spread to the peritoneal cavity?*

HGSC were found invading mesosalpinx, ovarian fat pad and spreading peritoneally (Figure 7e and f and Supplementary Figure 11b). We also provide description of such lesions in the text (page 12).

Reviewer #2 (Remarks to the Author):
The authors have largely addressed my comments.

We are glad that Reviewer #2 has been largely satisfied with our responses to his/her critiques.

1) For the 3 fibroblast populations shown in Figure 1, it would have been useful to at least mention them in the main text and highlight marker genes that were differentially expressed between them.

As advised by the Reviewer #2, we have mentioned marker genes differentially expressed among fibroblast populations in the main text (page 6 of the revised manuscript).

2) Similarly for the two Stem-epithelial in figure 1, it would have been useful to mention the markers that separate them. (e.g. are Slc1a3 and Cepd, differentially expressed between stem-epithelial 1 and steme-epithelial 2?).

We have mentioned markers distinguishing between the stem-like epithelial cell populations in the main text (page 6 of the revised manuscript).

3) It would also be useful to state which cells from figure 1 were taken forward into figure 2.

We have added a sentence to the legend of Supplementary Figure 1. We have additionally made it clearer within the text (pages 6 and 7 of the revised manuscript).

4) *I think the authors have done a good job at highlighting which populations are likely to be doublets. The inclusion of the fibroblast-like population in Fig2 doesn't really add anything to the story, but they have included the caveat that it is likely a doublet.*

We thank Reviewer #2 for a positive assessment of our previous revision.

Reviewer #3 (Remarks to the Author):
I would like to thank the authors for addressing all my comments. I am satisfied with their responses and the additional experiments they have included.

We thank Reviewer #3 for this positive assessment of our revisions.

1) I have one minor query concerning the origin of the peritoneal metastatic tumours produced in their Krt5 model in figure 7. There is an assumption that these originate from the premalignant lesions in the oviduct. Have the authors checked if their Creline is specific to the oviduct epithelium and does not hit cells in the ovarian surface epithelium, which could be an alternative source of these tumours. An experiment to show that their Krt5 model does not hit the ovarian surface epithelium and/or comparative genomic sequencing of the metastatic tumours with lesions in the oviduct from the same mouse would confirm their origin in the oviduct.

We have observed single Krt5+ cells in the ovarian surface epithelium (OSE) at 1 DPI. However, Krt5+ OSE cells were less frequent than those in the tubal epithelium, and their number decreases by 30 DPI. All mice in our study have been subjected to a thorough pathological evaluation of serial sections of both ovary and uterine tube (Methods, page 19 and 20 of the manuscript). No dysplastic OSE lesions were found in our studies of 24 mice between 90 and 427 DPI. We have added this information (new Supplementary Table 5). Comparative genomic sequencing has been insufficiently informative and produced contradictory results in previous studies of ovarian tumor

origin (e.g., Hao et l, Clin Cancer Res 23 (23): 7400-7411; Lawrenson et al., Cell Rep 29 (11): 3726-3735 e3724; McCool et al, Cancer Res 80 (4): 877-889). Thus, this approach is unlikely to help in our case.

I have also gone through reviewer 1s comments. In general, I think they authors have address most of their comments well. I would follow up on only two points:

We are thankful to Reviewer #3 for agreeing to assess our responses to Reviewer #1 and finding them largely satisfactory.

2) 1.5 - The MACS data is a more refined experiment and has gone someway to show the increased organoid forming potential of Slca1a3+ cells. However I do not think data fully supports their statement that Slca1a3+ cells are a subset of PAX8+ cells with hightened stem/progenitor characteristics. In their experiment they combine Slca1a3+ cells with a mixed epithelial population of Slca1a3- cells. This mixed population will contain multiciliated cells which are thought to be terminally differentiated and nonproliferative. Therefore the higher organoid forming capacity in Slca1a3+ can be interpreted as being a lower proportion of multiciliated cells rather than Slca1a3+ cells being a sub population of nonciliated cells with higher stem/progenitor characteristics. To fully support their claim that Slca1a3+ cells are a progenitor-stem cell they should also remove multiciliated cells from their experiment.

Based on Reviewer #3 comments we suspect that our description of MACS experiments have been insufficiently explanatory. These experiments do not have a goal of clarifying the relationship between PAX8+ and SLC1A3+ cells. We also do not combine Slc1a3+ cells with a mixed epithelial population of Slc1a3- cells. We have modified the Supplementary Table 3 and added a footnote to this table to make it clearer.

We are not sure how Reviewer #3 has arrived at the conclusion that removal of ciliated cells is required to claim that Slc1a3+ cells are "a progenitor-stem cell". As Reviewer #3 has correctly noted, ciliated cells are terminally differentiated and non-proliferative. Thus, their admix to organoid-forming Slc1a3+ cells would decrease not increase organoid formation frequency. We would like to note that organoid studies alone could only offer some information about differentiation potential of organoid forming cells, such as propensity for ciliation and secretion. Given significant plasticity of cells ex-vivo, organoid approaches would not be sufficient to conclusively establish stem/progenitor cell status and should be regarded as auxiliary. Our in vivo cell lineage studies, such as lineage trajectory prediction and cell fate tracing, are the main approaches establishing Slc1a3+ cells as progenitor-stem cells. We are not sure how to decide if some Slc1a3+ cells represent a lower proportion of ciliated cells rather than a sub population of non-ciliated cells with higher stem/progenitor characteristics. Based on our lineage trajectory studies, "a lower proportion" of ciliated cell trajectory and "higher portion" of stem/progenitor cells are likely to represent the same cell state. We hope that Reviewer #3 will agree that our current data lay a ground for future work on even more thorough analysis of cell states in the tubal epithelium.

3) Following on this point, in the authors response they point to scRNAseq data which shows that Slca1a3 labels a subset of PAX8 cells. This should be confirmed by immunofluorescence using PAX8 and Slca1a3 antibodies.

We have added this experiment (new Supplementary Figure 7).

4) 2.2 - A quantification of the total number of Tdt+ cells between days 1 and 30 would be helpful to show that Krt5+ cells are not expanding, thereby supporting their hypothesis that these are a transient cell population.

We respectfully disagree with Reviewer #3 that a transient state is expected to be non-proliferative. In many tissues transient cells are actively proliferating, thereby explaining their frequent name "transit-amplifying" cells. Best examples of such cells include the hair follicle, the hematopoietic system, the intestine, the nervous system, the corneal epithelium, and the male germline. Consistent with these observations, the total number of Krt5+ progeny is increasing. We have added this finding to the text and provide the quantification for 1 and 30 DPI (new Supplementary Table 5).

Point-by-point response to the reviewers' comments

Reviewer #3 (Remarks to the Author):

I would like to thank the authors for addressing all my comments and I agree that their findings supporting a "transient amplifying "hypothesis for Krt5+ cells.

We are pleased that Reviewer #3 shares our view on the properties of Krt5+ cells.

I suggest that a schematic summarizing the authors findings/thoughts on the epithelial cell hierarchy in the oviduct, including relevant markers would be helpful.

We have added a schematic suggested by Reviewer #3 as a Figure 8.

Also a statement outlining the limitations of their findings.

We have added a paragraph describing limitations of our findings (page 17 of the revised manuscript.

I do not think anymore experiments are required and would like to congratulate the authors for their excellent work.

We thank Reviewer #3 for this positive assessment of our work.