

Peer Review File

A cell atlas of the human fallopian tube throughout the menstrual cycle and menopause



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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The fallopian tube was an understudied organ for many years, but in the last two decades, our understanding of the fallopian tube as the home of the cell-of-origin for aggressive Mullerian cancers has motivated research into the cellular phenotypes and pathways governing typical homeostasis in this organ, and the perturbations that occur during disease development. In this report, Weigert et al use single cell transcriptomics and single cell epigenetics to dissect the dynamic responses of the fallopian tube to the hormonal changes that occur during the life course. This insight is very relevant for contextualizing studies into cancer origins, as many 'ovarian' cancer risk factors occur pre-menopausally, but tumors typically arise after menopause.

This is an important foundational study providing key insights into the cellular and molecular changes occurring in post-menopausal fallopian tubes and in the same organ during the menstrual cycle. These results will have value for the ovarian cancer community as well as others studying fallopian tube pathologies and reproduction. To add confidence to the new findings, key results are validated with orthogonal methodologies - spatial transcriptomics, IHC and in vitro co-cultures. Overall this is a solid study, well presented, with clearly explained figures and carefully compiled supplementary data. The authors could consider minor revisions to a couple of sections for clarity and/or to deepen the explorations, as follows-

1. Starting line 194 - It is tricky to follow how the SE-post-specific cluster was identified as this cluster is absent from some figures and associated supplementary tables (with cell numbers etc). Were the secretory cells reclustered to identify these cells? Please extend the description in the results or methods to assist the reader. It would also be beneficial to include details about how much each of the post-menopausal patients contribute to this cluster.

2. The altered epithelial-mesenchymal/immune interactions aspect is intriguing (Fig S2C), especially given the potential for important tumor suppressive signaling to be attenuated in the post-menopausal FT. The paper could benefit from further development of this aspect – are there any candidate interactions lost that the authors hypothesize might contribute to the changes in cellular composition and/or cancer risk (described at either the cell-cell level and/or ligand-receptor level).

In addition –

1. Confounding effect of age / race – this reviewer recognizes that resolving the impact of age and race are beyond the scope of this study, and that the inclusion of a racially diverse cohort is important to ensuring the results are relevant to non-Whites. But one challenge with smaller studies is that other variables may confound/interact with the variable of interest and we are underpowered to regress out those effects. For example here there is a strong association with menopausal status and age but each can have independent effects. It is also noted that the post-menopausal patients in this study are mostly White whereas 8/10 pre-menopausal patients are African American. The authors could potentially mine resources that catalogue age/race-related genes (eg the Aging Atlas) to disentangle age/race-related signals from the hormonally regulated features. However that is a significant undertaking and arguably beyond the focused scope of the study – ideally much larger studies will be needed in the future to fully disentangle the impact of these variables on this specific organ. However, it could be beneficial to include these caveats in the ‘Limitations of the study’ section.

2. Line 151-2 “decreased significantly... increased” suggest to add details of % change and p-value, for context. Other parts of the results that are also more qualitative/descriptive could benefit from additional the inclusion of quantitative information eg. Paragraph starting line 231

3. Please include detail of how many tissues were used for Visium, key patient epidemiologic data and how those data were analyzed after NGS, in the methods.

4. Please include, in the methods or corresponding legends, more detail about how the gene lists were curated for the GO analysis. Eg. ST9 – HLA-DRA appears as a DEG for SE1-pre and SE3-pre and is within the gene lists associated with enriched terms in the next tab. This same gene is not a DEG for SE-post but appears in 3 of the enriched GO terms for SE-post (lines 30, 32, 33).

5. Here existing data from post-menopausal samples are integrated with newly generated data for pre-menopausal samples. To minimize the possibility of batch effects the authors follow the same sample preparation, data generation and data analysis pipelines and integrate the data together using widely used methodologies for data integration. To further minimize any potential concern that differences in data metrics/technical artefacts could impact the results, it would be good to see whether there is a significant difference in QC metrics when the newly data are compared to the existing data (eg: mtRNA content, number of genes detected/cell, number of cells/sample, number of peaks/cell, number reads/cell). This information could be included as a supplementary figure.

6. In the supplementary table legends it would be helpful to have reminders of that the various abbreviations stand for

7. Figure S2B – please include a legend to explain what the different sizes of dots indicate

8. Figure S3D legend refers to p53 staining, which is not in the corresponding figure

Reviewer #2 (Remarks to the Author):

This manuscript presents a comprehensive investigation into age-related variations in human fallopian tubes (FT). The study employs a comparative analysis of cell abundance, differential gene expression (DGE), and cell regulation during both the menstrual cycle (n=85,107) and menopause (n=46,111). Previous research conducted this group (Lengyel et al., 2022) and others (Dinh et al., 2021; Ulrich et al., 2022; Hu et al., 2022) has significantly contributed to identifying various cell types in the fallopian tube, shedding light on cellular alterations associated with prevalent fallopian tube disorders.

Essentially, this study utilizes advanced single-cell techniques to explore the molecular-level effects of the menstrual cycle and menopause on diverse fallopian tube (FT) cells. The research additionally suggests potential markers in secretory epithelial cells that could be valuable for classifying ovarian cancer subtypes. However, it is crucial to acknowledge that the study is purely descriptive with no functional correlate. The main finding that is the understanding of the origin of high-grade serous cancer (HGSC) is poorly presented. To augment its comprehensiveness, it would be beneficial for the authors to incorporate additional analyses including the use of epithelial organoids that provide more insight into the functional alterations observed in FT cells.

Abstract

The abstract should be structured with information that is specific and relevant for the message communicated by the paper.

Results

Cohort description and Subject Details

1. Details regarding the cohort description and the collection of fallopian tube specimens should be included in the Methods section rather than the Results section. If you examine various anatomical areas, such as the isthmus, along with the ampulla and fimbriae, please ensure to incorporate them in the methodology as well.

2. While in the “cohort description”, authors indicate that fallopian tubes were collected from healthy female donors, the “subject details” section states that some patients had fibroids. Can you provide clarification on the specific exclusion and inclusion criteria and the methods used for determining them? Please indicate if subjects had bacterial, fungal, or viral infections (at the time of obtaining the specimens or a history of such in the past, since prior infection can alter the fallopian tubes features and function).

3. The BMI varies significantly among the donors, ranging from 19.55 to 53.34. How might this substantial difference impact the results?
4. The authors still using 'race' instead of 'ethnicity'? Is 'White' considered a race? What about Hispanic data—why is it separated from race? The manuscript does not provide information on these classifications or the rationale for separating them in this manner.
5. Figure 1B is not referenced in the text. It would be beneficial if the authors included a reference to this figure in the relevant sections. Additionally, it could be insightful if the authors included the same staining in a post-menopausal section to facilitate comparison and observation of differences.

Cellular composition of the pre-menopausal human fallopian tube.

1. When discussing about the identification of secretory epithelial cells in pre-menopausal samples, do you speculate whether this observation is authentic, or do you consider the possibility that it might be an artifact caused by the low cell count?
2. Line 108: Where did the authors obtain the information regarding canonical markers for pre-menopausal clustering? Was it from a database or previous studies? It is essential to include this information for clarity and reference.
3. Lines 113-124: It would be insightful for the authors to comment in the text on the varying percentages of populations observed in various types of tissues, as shown in FIGURE S1A/B.
4. From lines 140-144, the authors discuss the accessibility that different populations present to different TFs. It is recommended to explain the biological processes in which these TFs are involved and the importance of their action in different populations.

Human pre-menopausal and post-menopausal fallopian tubes differ in cellular composition and gene expression.

1. How did you analyse the cellular composition differences in FT samples before and after menopause? Did you employ a method based on cell proportions? I recommend utilizing the Milo approach outlined by Dann et al.,2022. This approach facilitates the grouping of cells on a k-nearest neighbour graph and assesses the variations in cell abundance between perimenopausal and postmenopausal states.
2. There is a limited amount of information available when examining interactions among different cell types using CellPhoneDB. Have you thought about exploring alternative methods, such as CellChat?

3. The observation of reduced stromal and immune cell interactions with aging is interesting. Is this phenomenon observed in other organs as well, suggesting a generalizable pattern?
4. When examining percentages in relation to pre-menopausal TF activities, you stated that androgen receptor exhibited an increase in ST3 and SM cell populations in post-menopause. Could you explain about the analysis to reach this result, please?
5. In the same context, you stated that estrogen receptor (ESR) activities increased in SE1? Could you please indicate which are these ESR activities and which genes are involved as well as confirm that they are increased in SE?
6. Line 160: The statement 'Several genes related to menopausal status' is made. Could the authors specify which genes and in which populations these genes are associated? Providing this information would enhance the understanding of the impact of menopause on the fallopian tubes.
7. It is challenging to comprehend why the authors present the differences in transcription factors in two ways, 3D and 3E, and why they differ. I would recommend that the authors provide additional reasoning for this choice and perhaps consider sticking with the analysis they deem superior for clarity and consistency.

Secretory epithelial cells change more significantly than other cell types between pre- and post-menopausal donor samples.

Overall, the authors' findings give rise to a crucial question: how the observed transcriptomic changes manifest as functional alterations in individual cell types? The authors might explore conducting functional assessments in SE cells using FT epithelial organoids and draw comparisons between pre-menopausal and postmenopausal samples or between proliferative and secretory phases.

1. Based on the UMAP profiling, SE post-menopausal cells seems to be the same, so what is the reason to divide them in two subtypes? Authors should provide a clearer explanation of the dissimilarities and validate that these distinctions are not merely artifacts arising from the integration process.
2. According to the data presented in Fig 4C, the proportion of cells expressing hormone receptors ESR1 and IGFIR is relatively consistent between pre- and post-menopausal samples. In contrast, there is a higher proportion of cells expressing ESR2 and LEPR in post-menopausal samples compared to pre-menopausal ones. Please review and modify the content of these paragraph: "In post-menopausal SE cells, the overall expression levels of hormone receptors are decreased but a small proportion of cells highly expressed ESR1, ESR2, IGFIR and LEPR". Further clarification of these results should be included in the discussion section.
3. What prompts the sudden examination of keratin when it hasn't been discussed in previous analyses? Additionally intriguing is the expression of TUBB in SE3 pre. Why not bring attention to it? What is your perspective on its potential role within this particular group?

4. Concerning the TF enrichment analysis, it would be convenient to show just one figure—either depicting TF activities or z-scores—because presenting both might lead to confusion in drawing conclusions.

5. The spatial transcriptome data in this research only capture alterations in certain genes in pre-menopausal samples. How many samples did you use? To appropriately validate the scRNA-seq findings, it is essential to incorporate spatial transcriptomics data from both pre- and post-menopausal samples to further investigate the spatial-specific changes during the menstrual cycle or during the aging process.. Kindly incorporate this information, if possible.

6. Line 238: Regarding spatial transcriptomics, from which samples were these analyses performed? Why is this validation limited to confirming scRNAseq data only? Spatial transcriptomics offers a wealth of information, and it would be beneficial to include more details about its application and insights gained.

During the menstrual cycle, secretory epithelial cells change more than other cell types

1. Statistical analysis should be conducted on the quantification of OVGP1 protein expression staining to determine the significance of differences between secretory and proliferative conditions.

2. What novelty arises when subjecting a co-culture of primary human stromal and epithelial cells, obtained from patients operated on for benign conditions, to stimulation with progesterone and estrogen treatment (E2)? The association between IGF1 and IGF2 after hormonal treatment has already been documented.

3. Line 254: The images in Figure S4C seem to show a high presence of OVGP1 in one of the progesterone and one of the postmenopausal images. This observation contradicts the described result in the text, making it inappropriate to assert that SE1-pre cells are regulated by progesterone.

4. Line 254: The statement that SE2-pre cells and OVGP1 expression are estrogen-regulated is made, but it should be noted that this is a suggestion based on indirect results of the analysis (FIG 5C-D and S4B) and lacks validation.

5. Line 283: Why RNA velocity was not performed on post-menopausal samples? Including these samples could reveal a drift from pre-menstrual to post-menstrual populations. It would also be interesting to conduct RNA velocity analysis on all cell types in premenopausal samples, not just SE.

Secretory epithelial cell markers can subtype high grade serous ovarian cancer

1. This section is the most relevant and novel of the paper. The deconvolution analysis presented in figure 6, should be the basis to prove the functional relevance of this finding using an in vitro model of FT epithelial organoids, otherwise it will remain as descriptive and speculative.

Methods

1. What is the resolution for clustering? A statistical approach to identify the resolution or find the number of clusters should be performed.

2. How were batch effects addressed when combining single-cell RNA sequencing (scRNAseq) and single-cell ATAC sequencing (scATACseq), or when merging databases from pre- and postmenopausal samples? Did the correction involve the inclusion of a design matrix in the scaling procedure, and were the influences of cell cycle genes eliminated through regression?

3. The data preprocessing pipeline outlined in the Methods section, named 'scRICA,' is based on the GitHub and R Vignettes and has been developed by the authors. However, it has not been formally published to date. The manuscript should provide a detailed description of the procedures, explicitly stating whether the CellRanger suite was utilized for certain preprocessing quality controls and any subsequent implemented steps. Additionally, it is crucial to disclose the versions of packages and software used in this pipeline

4. There is no reference to ambientRNA correction, a well-known artifact that significantly influences the downstream interpretation of results. The authors should provide evidence of this quality control (QC) step, along with details on the other QC-based filters applied. Including a table specifying the number of cells filtered at each step would enhance the transparency of the methodology.

5. Some results indicate variations in cellular abundances between pre- and postmenopausal women. The approach taken to assess differential abundances involves conducting a t-test comparison between the number of cells in one condition versus the cells in the other condition. However, this analysis fails to consider the topology of observations in the transcriptomic space beyond the assigned cell type tags. It is crucial to note that differential cell abundance analysis should ideally be conducted in an unsupervised manner. Cells are distributed along a continuum, and as such, there may be overlooked regions of different abundances (e.g., smaller, larger, or between clusters). To fully harness the potential of single-cell data, a more nuanced analysis is recommended. Numerous studies explore differential abundances between conditions, and there are several published methods that could enhance the analysis. I suggest considering at least one of these methods to accurately assess regions of differential abundance in the data.

6. Line 578 states, 'We summarized cell numbers based on cell annotations and normalized the relative cell percentage by the total number of cells. We only reported significance with $p < 0.05$.' Could you please clarify if this statement pertains to Cell Abundance analysis? Kindly provide an explanation and state this explicitly.

7. The Methods section also lacks the application of advanced tools for RNA velocity analysis. While Velocityto is a powerful tool, it is recognized that inference may be biased by unmodeled transcription kinetics (REF. Bergen V, Soldatov RA, Kharchenko PV, Theis FJ. RNA velocity-current challenges and future perspectives. Mol Syst Biol. 2021 Aug;17(8):e10282. doi:

10.15252/msb.202110282. PMID: 34435732; PMCID: PMC8388041). To enhance the reliability of their results, the authors should address this potential bias in their analysis.

8. Furthermore, the analysis lacks some discussion and graphical support for the gene expression of features that drive the overall kinetics and the patterns supporting the obtained trajectory. Incorporating this information could offer crucial biological insights and provide additional support for the presented results.

9. The integrative analysis of scRNAseq and scATACseq was conducted using transfer anchors (refer to the pipeline for details) to annotate cell types. Cells with a prediction score greater than 0.5 were retained. Could the authors provide an explanation for the choice of this cutoff, as opposed to, for instance, 0.7, which might yield more reliable results?

Discussion

- In the discussion (lines 323-334), the authors discuss the presence of a "SOX9+, LGR5+" population. If these markers are considered relevant, they should be introduced earlier in the text, which is not currently done. Additionally, the SE2 population appears to be substantial for a somatic stem cell population, and the authors do not provide references justifying the existence of such a population in the fallopian tubes.

-Line 330: In the endometrium, SOX9+ and LGR5+ cells have been proposed as stem/progenitor cells, representing a small fraction within the analyzed epithelial cells in the corresponding study. However, in this case, SE2 cells constitute 84% of SE cells in the pre-menopausal sample. What could explain such a large stem population in the fallopian tube?

- SOX9 is not mentioned in the results, and it is the only gene (along with LGR5) that appears in the discussion of healthy populations with no relation to cancer.

-Have the authors examined the expression of SOX9 and LGR5 genes in epithelial cells throughout their visium experiments to assess whether the populations spatially behave as previously described for the endometrium (as referenced in this paper)?

- In general, the manuscript frequently mentions genes and factors, either for defining populations or due to differential expression. However, the authors should provide more comprehensive explanations of the biological implications of these genes to enhance the understanding of fallopian tube functioning, accompanied by relevant references.

- I recommend a more thorough exploration of the results in the discussion section. A detailed analysis and interpretation of the findings would significantly enhance the overall comprehension of your research. Additionally, consider incorporating references that support and strengthen your results.

Minor comments

Table S1. What is the meaning of N/A In relation with the TF enrichment analysis, it would be convenient to show only one figure (either TF activities or z-score) since the conclusion could be confused? It denotes either not applicable or not available. Please clarify.

Figure 1A and Figure S4A both depict the uterus throughout the menstrual cycle with the same content. It is advisable to retain one of these figures and eliminate the redundancy.

Figure S2.B: Some gene symbols are overlapping, making it difficult to read them.

Figure S3.D: Heatmap titles are at different heights. Try to plot them more uniformly.

Figure 2.D.: Same as S3.D.

Fig 4A: Are 58 cells enough to determine a cell cluster???

Figure 4.D.: Same as S3.D and 2.D.

Fig 5E, 5C, 5D. To enhance the reviewer's understanding and facilitate future readers' comprehension, it is recommended that the sequence of figures aligns consistently with the corresponding explanations throughout the manuscript.

Figure 6.A-B.: Same as S3.D, 2.D, and 4.D. "High-grade serous Ovarian Cancer" is the title of 6B heatmap. Please include a descriptive title.

Line 116: replace "Table 4" for "Table S4"

Lines 163 and 164: Why are those markers associated with each function? Could the authors provide references supporting these associations?

Please indicate the versions of the software used to perform analyses: Line 485 "scRICA", Line 488 "DoubletDecon", Line 494 "HIPPO", and Line 515 "clusterProfiler".

Please specify the specific software used to perform the integration of scRNAseq and ATACseq datasets.

Regarding the single-cell analysis packages, some are in italics (DoubletDecon at Line 488), and the others are not (scRICA at Line 485, HIPPO at Lines 494 and 496, ...). Try to standardize this.

There are unnumbered references in methods. Please pass all references to the corresponding numbers."

Reviewer #3 (Remarks to the Author):

The Fallopian tubes are the major site of origin of ovarian cancer, which had motivated an increasing number of studies to understand the physiology of this tissue and the mechanisms triggering the development of ovarian cancer. In this paper, Weigert et al look at changes in expression pattern that occur with menopause, as well as during the menstrual cycle. To achieve this, the authors performed single cell transcriptomics and single cell chromatin accessibility profiling. This study can help to understand changes occurring in healthy women during their lifetime and insights in the different events occurring during cancer development, including early

event occurring before menopause but potentially rendering the tissue more susceptible to neoplastic transformation later in life.

This work is of great interest for the ovarian cancer community as well as other scientists working on the Fallopian tubes. This study shows strong evidence of the results claimed, reinforced by additional experiments like spatial transcriptomics and IHC. Results are well presented, and legends are clear enough to understand the results shown. Authors could revise the following point to a better understanding and increase the robustness of their results:

- SE post specific cluster: It is difficult to understand how this cluster has been identified. The markers of secretory epithelial cells mentioned in the table S3 doesn't seem to be expressed on this specific cluster. Additional details on markers used to classify these cells as secretory will be beneficial. Indicating the number of cells in this specific cluster for each sample could also help to have a better view of the robustness of the analysis.

- Menstrual cycle phase analysis: the number of samples per group is small, limiting the power to detect associations. Therefore, while difficult to do, you could consider increasing the number of samples to increase the power of the analysis.

Minor :

- Fig3A: you mention 11 women for premenopausal group while you have 10 patients

- line 119: "We also separated EN cells....based on expression pattern of KDR and CLDN5" this is confusing, clusters are made by the unsupervised clustering analysis and the expression pattern in these sub-clusters is different.

- Fig3A and 5A: For this type of proportion comparison it could be very beneficial to add the individual value on top of the box plot. This could help to see any samples strongly divergent or detect the presence of different groups of samples. Overall adding this information increases the confidence in the analysis.

- SE3 cluster: you say that there are only 58 cells in this cluster but then perform a differential expression analysis on these cells. This analysis is very difficult to trust especially because more

than half of these cells come from 2 samples. You should either provide more detail, especially on QC on these cells or remove the analysis of this cluster.

- Confounding effect: It is recognized that having a cohort with samples coming from very similar patient is challenging. However, in this paper the two main group differ largely in term of race as 6/7 patient of postmenopausal are white while 8/10 of the premenopausal group are African American. Although it's not clear how the race could affect the expression pattern of the Fallopian tubes, we can't exclude an impact of this on the results of this study. Therefore, this limitation should be discussed.

- Fig5C: It is difficult to understand why you compared the secretory and proliferative phase by using two different clusters (Secretory SE1 vs proliferative SE2). The results should explain more clearly this choice and the impact of the analysis.

- Sequencing information: In this paper new data are integrated with old data. While this is totally acceptable, more information on the QC of each sample should be provided to determine if the two dataset are comparable or not. This information can be added to a supplement figure and or table. Adding others relevant information about the sequencing might be considered, especially because 2 different sequencers and different number of cycles has been used for this paper.

- Visium: This experiment is useful to increase the confidence in the results. However this section lacks key experimental detail on how many samples (and which ones) has been analyzed by this spatial transcriptomic analysis.

- Code: In study including computational analyses the code should be provide as a method (github link) to allow the reproduction of the results

Reviewer #4 (Remarks to the Author):

This is an impressive and important manuscript that catalogues the single cell transcriptomes of the fallopian tube in premenopausal and postmenopausal women. The finding are important and complement previous reports from the authors themselves as well as previously published single cell data sets on the fallopian tube, the most likely tissue of origin of serous ovarian cancer.

My minor concern relates to figure 6. I found it hard to understand the findings and relevance of figure 6A. No quantification of the degree of overlap between the single cell data and the TCGA data in multiple tumours. Not sure what is the relevance of comparing the data with breast cancer data for example. I suggest either removing this all together or moving it to supplementary. Also, figure 6B and 6C are interesting but the classification of ovarian tumours presented by TCGA is interesting. However, a more recent classification (PMID: 32049047) with robust prognostic significance based on FT single cell sequencing would be highly relevant to this work. It would be great if this classification was considered instead of or along with the TCGA classification in figures 6B and 6C.

REVIEWER COMMENTS

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The fallopian tube was an understudied organ for many years, but in the last two decades, our understanding of the fallopian tube as the home of the cell-of-origin for aggressive Mullerian cancers has motivated research into the cellular phenotypes and pathways governing typical homeostasis in this organ, and the perturbations that occur during disease development. In this report, Weigert et al use single cell transcriptomics and single cell epigenetics to dissect the dynamic responses of the fallopian tube to the hormonal changes that occur during the life course. This insight is very relevant for contextualizing studies into cancer origins, as many 'ovarian' cancer risk factors occur pre-menopausally, but tumors typically arise after menopause.

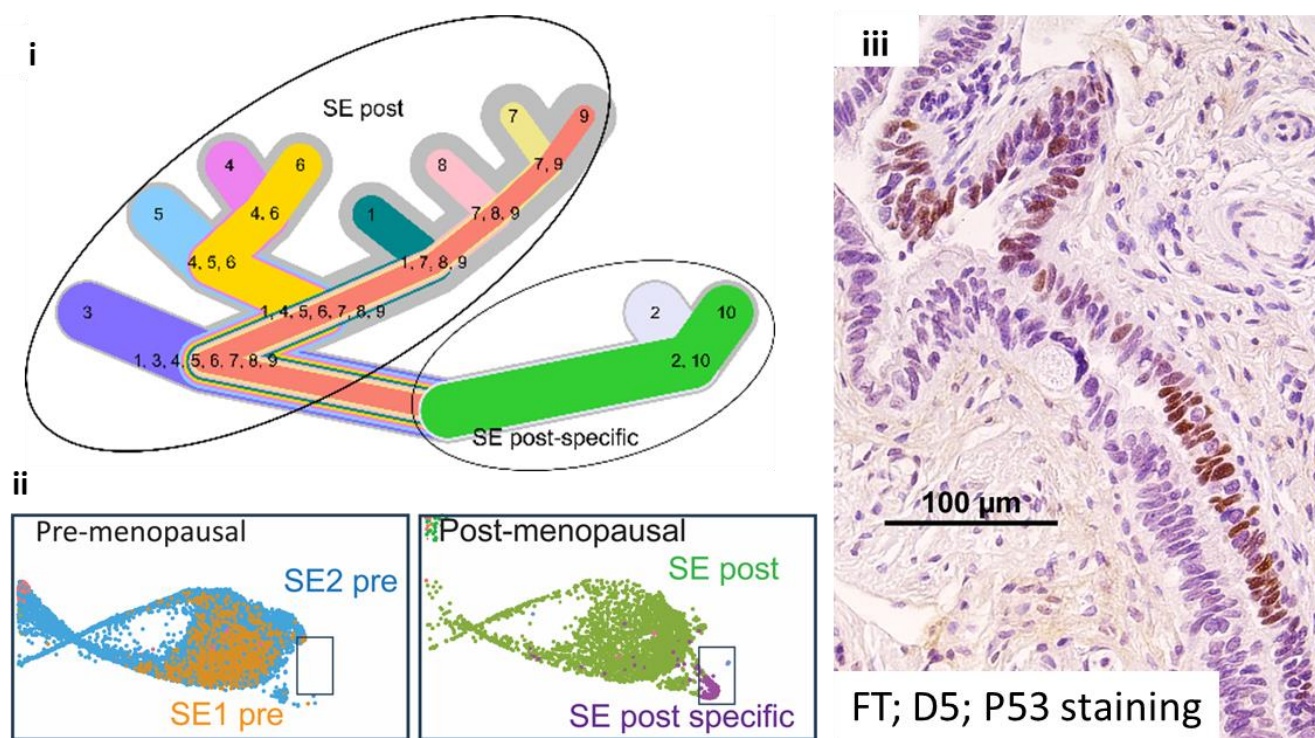
This is an important foundational study providing key insights into the cellular and molecular changes occurring in post-menopausal fallopian tubes and in the same organ during the menstrual cycle. These results will have value for the ovarian cancer community as well as others studying fallopian tube pathologies and reproduction. To add confidence to the new findings, key results are validated with orthogonal methodologies - spatial transcriptomics, IHC and in vitro co-cultures. Overall, this is a solid study, well presented, with clearly explained figures and carefully compiled supplementary data. The authors could consider minor revisions to a couple of sections for clarity and/or to deepen the explorations, as follows-

1. Starting line 194 - It is tricky to follow how the SE-post-specific cluster was identified as this cluster is absent from some figures and associated supplementary tables (with cell numbers etc.). Were the secretory cells re-clustered to identify these cells? Please extend the description in the results or methods to assist the reader. It would also be beneficial to include details about how much each of the post-menopausal patients contribute to this cluster.

We apologize for any confusion caused by our previous submission. To clarify, our last submission included both figures and tables dedicated to the analysis of pre-menopausal samples exclusively, as well as those representing a combination of pre- and post-menopausal samples. In response to your comments, we have added explicit descriptions in the main text and method section regarding the identification of a post-menopausal specific SE cluster.

To identify subtypes within postmenopausal SE cells, we applied a hierarchical clustering approach named HIPPO, which is based on zero proportions from UMI count data (refer to Rebuttal Figure 1A). Subsequently, we integrated the results from the HIPPO analysis with UMAP visualizations of both pre- and post-menopausal SE cells. This analysis revealed a distinct major cluster branch, incorporating sub-clusters 2 and 10 (**Fig. A-i**), which did not coincide with any clusters identified in pre-menopausal SE cells (**Fig. A-ii**). We performed p53 IHC and did indeed find fallopian tube epithelial cells with positive p53 staining (**Fig. A-iii**) We have designated this unique cluster branch as the SE-post-specific cluster.

Furthermore, to provide a comprehensive view of the data, we have included a detailed summary of the contributions from each post-menopausal patient in the manuscript supplementary table 2. We trust that these amendments will enhance the clarity and understanding of our findings.



Rebuttal Figure A: i) HIPPO clustering of SE-postmenopausal sample clustering results. ii) UMAP of pre- and postmenopausal SE cells highlighting the SE-post specific cell cluster. iii) P53 immunohistochemistry of the fallopian tube fimbriated end of postmenopausal patient D5.

2. The altered epithelial-mesenchymal/immune interactions aspect is intriguing (Fig S2C), especially given the potential for important tumor suppressive signaling to be attenuated in the post-menopausal FT. The paper could benefit from further development of this aspect – are there any candidate interactions lost that the authors hypothesize might contribute to the changes in cellular composition and/or cancer risk (described at either the cell-cell level and/or ligand-receptor level).

We thank the reviewer for highlighting the potential implications of altered epithelial-mesenchymal/immune interactions in the post-menopausal fallopian tube (FT). In the secretory phase of the menstrual cycle, high progesterone levels are known to inhibit uterine epithelial cell proliferation and foster an immunotolerant environment facilitated by Treg cells (PMID: 37191999). Furthermore, progesterone influences stromal cell behavior, which in turn affects epithelial-stromal communication critical for embryo implantation.

Our data align with these findings, indicating robust epithelial-stromal crosstalk during the secretory phase. Notably, we documented a surge in ligand-receptor interactions, particularly between SE1-pre and ST2 cells, within the proliferative phase. SE1-pre cells appear to activate pathways associated with steroid hormone regulation, including MHC class II processing, which suggests an immune modulatory role (PMID: 37191999, 30272581).

Addressing the specific query regarding post-menopausal FT, our observations suggest diminished stromal/epithelial interactions, which we hypothesize is attributable to the decreased hormonal influence characteristic of the post-menopausal milieu. This reduction could potentially lead to a lowered efficacy in tumor-suppressive signaling pathways. While we have not identified specific lost interactions, our data point towards a broad attenuation of the cellular communication networks that could contribute to altered cellular composition and increased cancer risk post-menopausal. This aspect presents a compelling direction for future research to identify the molecular interactions that may be compromised and their implications for cancer biology.

In addition,

1. Confounding effect of age / race – this reviewer recognizes that resolving the impact of age and race are beyond this study's scope, and that including a racially diverse cohort is important to ensuring the results are relevant to non-Whites. But one challenge with smaller studies is that other variables may confound/interact with the variable of interest, and we are underpowered to regress out those effects. For example, here there is a strong association with menopausal status and age, but each can have independent effects. It is also noted that the post-menopausal patients in this study are mostly White whereas 8/10 pre-menopausal patients are African American. The authors could potentially mine resources that catalogue age/race-related genes (e.g. the Aging Atlas) to disentangle age/race-related signals from the hormonally regulated features. However, that is a significant undertaking and arguably beyond the focused scope of the study – ideally much larger studies will be needed in the future to fully disentangle the impact of these variables on this specific organ. However, it could be beneficial to include these caveats in the 'Limitations of the study' section.

Thank you for your insightful suggestions. We have added these considerations in the 'Limitations of the study' section. We have elaborated on the potential confounding effects of ethnicity in our study by noting the racial composition of our cohorts: *"The potential confounding effect of ethnicity is acknowledged, as the majority of the postmenopausal cohort (six out of seven) were Caucasian, while the premenopausal cohort predominantly comprised African American individuals (eight out of ten)."*

2. Line 151-2 "decreased significantly... increased" suggest to add details of % change and p-value, for context. Other parts of the results that are also more qualitative/descriptive could benefit from additional the inclusion of quantitative information e.g. Paragraph starting line 231.

Given the high variability in cell numbers between patients, presenting percentage changes may not provide additional clarity and could potentially be misleading. P-values have been reported in the figure legends where relevant. For discussions such as those starting at Line 231 pertaining to Manuscript Figure 4G, we argue that including extensive quantitative data within the narrative would be superfluous and may detract from the readability, as the associated figures sufficiently convey this information.

3. Please include detail of how many tissues were used for Visium, key patient epidemiologic data and how those data were analyzed after NGS, in the methods.

As requested, we have now incorporated detailed information on the tissue sample utilized for Visium analysis, and the subsequent analysis methods into the methods section of our manuscript. Spatial transcriptomics was performed on a separate pre-menopausal patient (D19; amended table S1). We also expanded the methods section: *"The data was then loaded using Load10X Spatial function in R Seurat and normalized using SCTransform. The SpatialFeaturePlot function was applied to visualize the gene expression in the histology image."*

4. Please include, in the methods or corresponding legends, more detail about how the gene lists were curated for the GO analysis. E.g. ST9 – HLA-DRA appears as a DEG for SE1-pre and SE3-pre and is within the gene lists associated with enriched terms in the next tab. This same gene is not a DEG for SE-post but appears in 3 of the enriched GO terms for SE-post (lines 30, 32, 33).

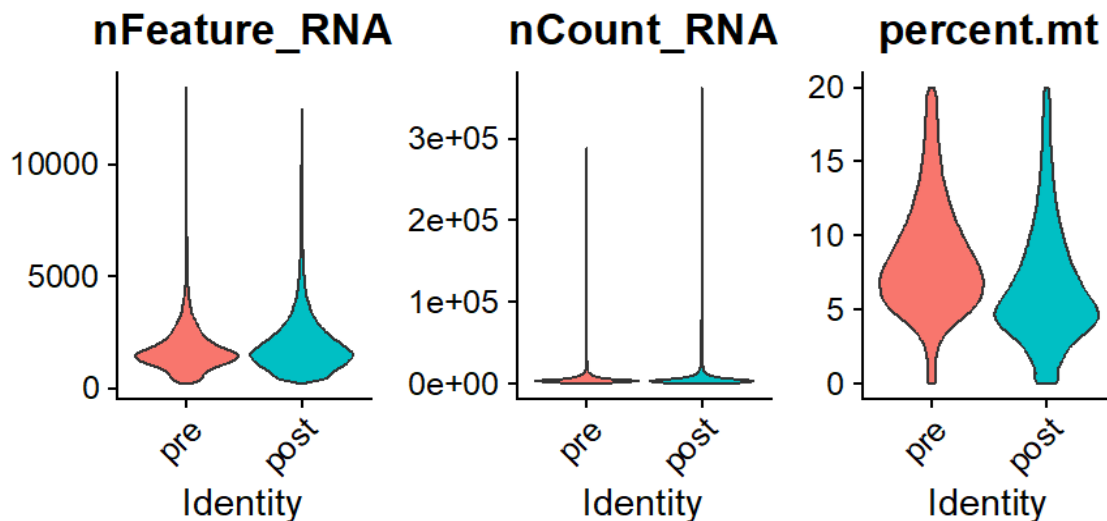
Our criteria for selecting the GO terms highlighted in the figures were based on their putative mechanistic relevance for fallopian tube function in both pre- and postmenopausal women, as per the full GO database (PMID: 10802651, 36866529). This explains the presence of genes such as HLA-DRA, which appears in the gene lists associated with enriched terms, despite not being differentially expressed in the SE-post group.

5. Here existing data from post-menopausal samples are integrated with newly generated data for pre-menopausal samples. To minimize the possibility of batch effects the authors follow the same sample preparation, data generation and data analysis pipelines and integrate the data together using widely used methodologies for data integration. To further minimize any potential concern that differences in

data metrics/technical artefacts could impact the results, it would be good to see whether there is a significant difference in QC metrics when the newly data are compared to the existing data (e.g.: mtRNA content, number of genes detected/cell, number of cells/sample, number of peaks/cell, number reads/cell). This information could be included as a supplementary figure.

Thank you for emphasizing the importance of rigorous quality control (QC) measures, particularly when integrating existing post-menopausal data with newly acquired pre-menopausal data.

Responding to your recommendation, we have conducted a comprehensive comparison of QC metrics between the two datasets, including mitochondrial RNA (mtRNA) content, the number of genes detected per cell, and the number of reads per cell (**Fig. B**). Our findings confirm that there is no substantial difference in these QC metrics between the pre- and post-menopausal groups. These results are now presented in supplementary table S2. We believe that the inclusion of these QC comparisons further substantiate the integrity of our data and the conclusions drawn from our study.



Rebuttal Figure B: QC metrics for data integration showing comparisons for features, transcript counts and mitochondrial DNA across cohorts.

6. In the supplementary table legends it would be helpful to have reminders of that the various abbreviations stand for.

We added a tab with all abbreviations for each supplementary table.

7. Figure S2B – please include a legend to explain what the different sizes of dots indicate

We included the following in Fig. S2B legend: “*Bigger dot sizes highlight the position of genes of interest.*”

8. Figure S3D legend refers to p53 staining, which is not in the corresponding figure

The incorrect panel description was removed.

Reviewer #2 (Remarks to the Author):

This manuscript presents a comprehensive investigation into age-related variations in human fallopian tubes (FT). The study employs a comparative analysis of cell abundance, differential gene expression (DGE), and cell regulation during both the menstrual cycle (n=85,107) and menopause (n=46,111). Previous research conducted this group (Lengyel et al., 2022) and others (Dinh et al., 2021; Ulrich et al., 2022; Hu et al., 2022) has significantly contributed to identifying various cell types in the fallopian tube, shedding light on cellular alterations associated with prevalent fallopian tube disorders.

Essentially, this study utilizes advanced single-cell techniques to explore the molecular-level effects of the menstrual cycle and menopause on diverse fallopian tube (FT) cells. The research

additionally suggests potential markers in secretory epithelial cells that could be valuable for classifying ovarian cancer subtypes. However, it is crucial to acknowledge that the study is purely descriptive with no functional correlate. The main finding that is the understanding of the origin of high-grade serous cancer (HGSC) is poorly presented. To augment its comprehensiveness, it would be beneficial for the authors to incorporate additional analyses including the use of epithelial organoids that provide more insight into the functional alterations observed in FT cells.

Abstract

The abstract should be structured with information that is specific and relevant for the message communicated by the paper.

[We included more relevant information in the revised abstract.](#)

Results: Cohort description and Subject Details

1. Details regarding the cohort description and the collection of fallopian tube specimens should be included in the Methods section rather than the Results section. If you examine various anatomical areas, such as the isthmus, along with the ampulla and fimbriae, please ensure to incorporate them in the methodology as well.

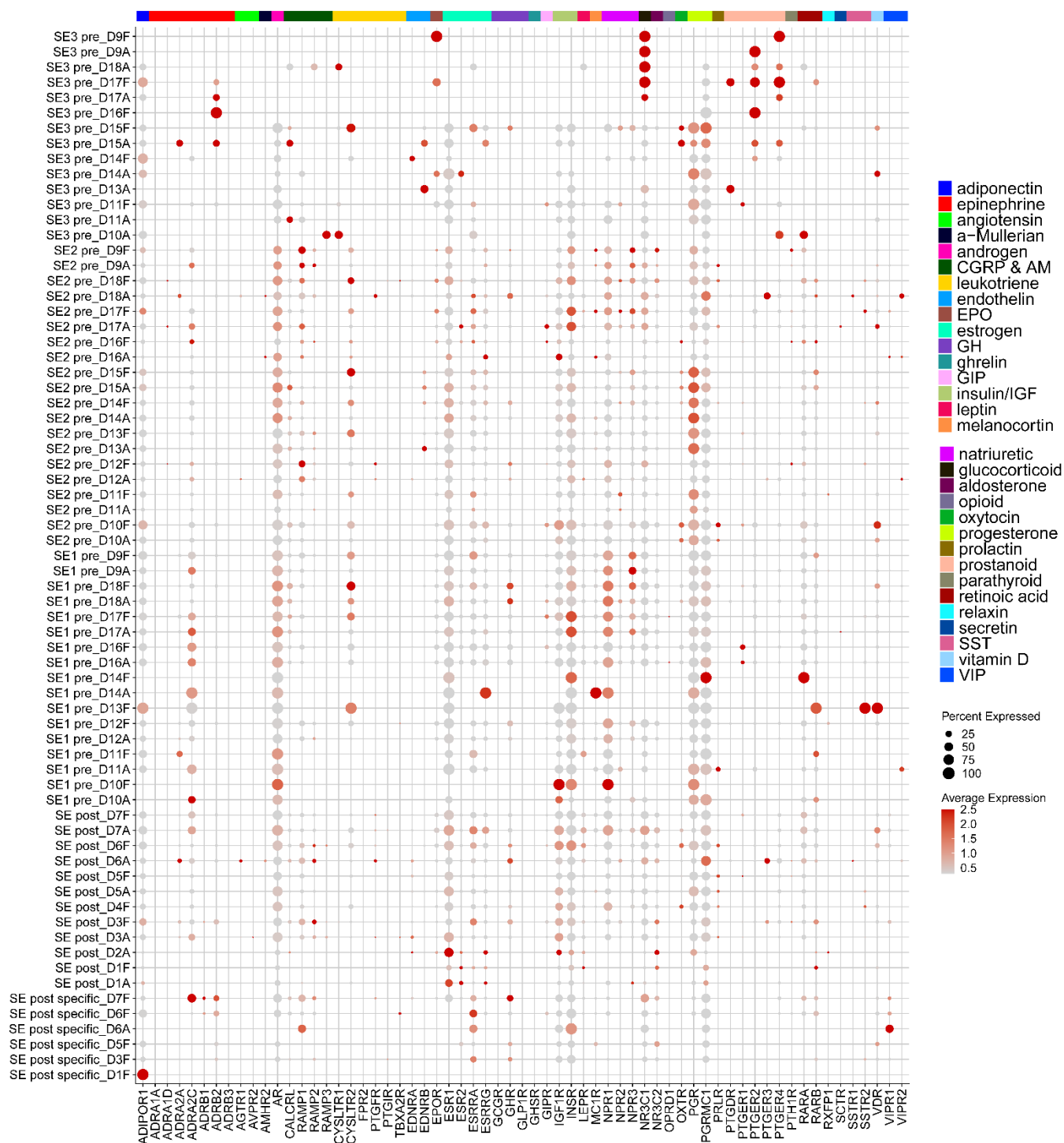
[We moved the cohort description and fallopian tube collection to the methods section.](#)

2. While in the “cohort description”, authors indicate that fallopian tubes were collected from healthy female donors, the “subject details” section states that some patients had fibroids. Can you provide clarification on the specific exclusion and inclusion criteria and the methods used for determining them? Please indicate if subjects had bacterial, fungal, or viral infections (at the time of obtaining the specimens or a history of such in the past, since prior infection can alter the fallopian tubes features and function.

[We have provided clarification on the exclusion and inclusion criteria for the study. Specifically, women without macroscopic or microscopic \(H&E\) fallopian tube pathologies were considered as healthy for this study. We added a column about previous history of gynecology-associated infections to Table S1.](#)

3. The BMI varies significantly among the donors, ranging from 19.55 to 53.34. How might this substantial difference impact the results?

[Within our patient cohort the BMI of two patients was above one standard deviation \(D10, D17\) and of two patients below one standard deviation \(D4, D15\) \(AV: 29.9, STDEV: 7.55\). It has been well described that BMI affects hormone signaling \(menstrual cycle irregularities\). In our study three BMI outliers were part of the pre-menopausal cohort, but all three patients had regular menses, therefore we assume that BMI in this case did not affect cycle length. We also looked at hormone receptor expression within SE cell population by patients and anatomical site and did not observe any significant differences in hormone receptor signaling associated with BMI \(Fig. C\).](#)



Rebuttal Figure C: Patient-specific hormone receptor signaling in all SE cell clusters.

4. The authors still using 'race' instead of 'ethnicity'? Is 'White' considered a race? What about Hispanic data—why is it separated from race? The manuscript does not provide information on these classifications or the rationale for separating them in this manner.

Thanks for pointing this out. We amended Table S1 accordingly.

5. Figure 1B is not referenced in the text. It would be beneficial if the authors included a reference to this figure in the relevant sections. Additionally, it could be insightful if the authors included the same staining in a post-menopausal section to facilitate comparison and observation of differences.

We now referenced Manuscript Figure 1B. We did not include the identical H&E staining for post-menopausal samples, because it would be difficult to quantify difference on H&E between pre- and postmenopausal fallopian tube cell populations.

Cellular composition of the pre-menopausal human fallopian tube.

1. When discussing about the identification of secretory epithelial cells in pre-menopausal samples, do you speculate whether this observation is authentic, or do you consider the possibility that it might be an artifact caused by the low cell count?

Secretory epithelial cells have been well described in the fallopian tube epithelium of premenopausal women (PMID: 7714136). We also highlighted the presence of secretory cells in Figure 1B (H&E). Given that we found over >11,000 cells we do not believe that this is an artifact (Rebuttal Table 1, below). SE cells were the 3rd most abundant cell type in our analysis after stromal and T/NK cells. Moreover, a recent manuscript also found secretory epithelial cells in pre-menopausal women (PMID: 35320732).

Cluster	Full name	Total # of cells in cluster
SE1	Secretory epithelial cells subtype 1	1,675
SE2	Secretory epithelial cells subtype 2	9,787
SE3	Secretory epithelial cells subtype 3	58

Rebuttal Table 1: Numbers of secretory cells in each pre-menopausal SE subcluster.

2. Line 108: Where did the authors obtain the information regarding canonical markers for pre-menopausal clustering? Was it from a database or previous studies? It is essential to include this information for clarity and reference.

Canonical markers for cells for the human postmenopausal fallopian tube (Lengyel et al., 2022) and premenopausal fallopian tube (Ulrich et al., 2022) were used. We added all references for further clarity in the manuscript text.

3. Lines 113-124: It would be insightful for the authors to comment in the text on the varying percentages of populations observed in various types of tissues, as shown in FIGURE S1A/B.

We added the following in the revised version: *“Comparison of the three different FT anatomical sites, showed similar cell distributions for almost all cell types. The isthmus had the highest percentage of SM cells and lowest percentages of CE and SE cells consistent with its function transporting the ovum towards the uterine cavity. In the fimbriae the prevalent cells are the CE and SE cells consistent with the large surface area required to increase the chances of the egg being picked up by the fallopian tube (Fig. S1B).”*

4. From lines 140-144, the authors discuss the accessibility that different populations present to different TFs. It is recommended to explain the biological processes in which these TFs are involved and the importance of their action in different populations.

We added more detailed descriptions for some of the biological processes of TFs described but had to adhere to word limits.

Human pre-menopausal and post-menopausal fallopian tubes differ in cellular composition and gene expression.

1. How did you analyze the cellular composition differences in FT samples before and after menopause? Did you employ a method based on cell proportions? I recommend utilizing the Milo approach outlined

by Dann et al.,2022. This approach facilitates the grouping of cells on a k-nearest neighbour graph and assesses the variations in cell abundance between perimenopausal and postmenopausal states.

We used t-test and proportion test to assess the difference. While we did observe variations in cellular abundances, we exercised caution in interpreting these data due to the well-documented limitations of single-cell transcriptomics in accurately reflecting true cellular abundance. These limitations stem from the potential for technical and experimental biases to influence the data.

Considering this, our analytical approach was not solely reliant on assessments of cell proportions. Instead, we focused primarily on differential gene expression analysis. This method, which benefits from the rich, multidimensional dataset provided by thousands of gene expressions, offers a deeper and more reliable insight into the biological shifts occurring across menopausal transitions.

2. There is a limited amount of information available when examining interactions among different cell types using CellPhoneDB. Have you thought about exploring alternative methods, such as CellChat?

Thank you for suggesting alternative methods for analyzing cell-cell interactions, such as CellChat. We acknowledge that recent studies (PMID: 35680885, 35822343) have highlighted the importance of method and resource selection in the inference of cell-cell communication, noting that these choices can significantly affect the outcomes of such analyses. Despite the availability of various methodologies for assessing cell-cell communication, we maintained consistency with our previous work on the post-menopausal cohort (PMID: 36543131) by employing the same methodological approach, specifically CellPhoneDB. This decision ensured comparability and continuity between the studies, allowing for a coherent analysis across the different cohorts.

3. The observation of reduced stromal and immune cell interactions with aging is interesting. Is this phenomenon observed in other organs as well, suggesting a generalizable pattern?

In our study we only focused on the human fallopian tube and have not investigated these findings in other organs, therefore we can only speculate on its generalizability. We speculate that the reduced immune and stromal/ epithelial interactions are due to the cessation of menstruation, which may not affect other organs to the same extent. Furthermore, stromal and epithelial immune cell interactions are critical to promote immune tolerance.

4. When examining percentages in relation to pre-menopausal TF activities, you stated that androgen receptor exhibited an increase in ST3 and SM cell populations in post-menopause. Could you explain about the analysis to reach this result, please?

We performed two types of analysis to compare TF activities in pre- and post-menopausal women. The first heatmap (Manuscript Fig. 3D) illustrates the absolute differences in selected transcription factor activities by cell type. Here, the z-score difference is calculated by directly subtracting the average pre-menopausal TF activity from the post-menopausal activity, which we refer to as (Post - Pre). This approach provides a straightforward comparison of TF activities between the two states.

The second heatmap (Manuscript Fig. 3E) represents the relative changes in transcription factor activities by cell type, expressed as a percentage change from the pre-menopausal levels $((\text{Post} - \text{Pre}) / \text{Pre} * 100)$. We included this analysis to ensure that TFs with substantial relative changes, which may not be apparent when looking at absolute differences alone due to their low baseline activities, are not overlooked. This method highlights TFs that may have a significant biological impact, even if their absolute activity changes are small. On this heatmap, in post-menopausal donors, the androgen receptor (AR) and the glucocorticoid receptor (NR3C1) exhibited an increase in ST3 and SM; MAFK and MAFF displayed an increase in all cell types except T/NK, EN1, P/V1, and B/P1; The estrogen receptor (ESR) family activities increased in SE1 and MA; TWIST1 increased in ST1 and MP.

5. In the same context, you stated that estrogen receptor (ESR) activities increased in SE1? Could you please indicate which are these ESR activities and which genes are involved as well as confirm that they are increased in SE?

We stated in our manuscript that the EGR family activities (not ESR) are increased in SE1 and MA clusters post-menopausal (Fig. 3E).

6. Line 160: The statement 'Several genes related to menopausal status' is made. Could the authors specify which genes and in which populations these genes are associated? Providing this information would enhance the understanding of the impact of menopause on the fallopian tubes.

We provide the requested information in the following sentences:

“Several genes were specific to menopausal status. PCSK1N (SM and P/V1-2), OVGP1 (SE cells), CRISP3, and CYBA (SE cells, B/P1-2, and immune cells), exhibited significantly higher expression levels in pre-menopausal compared to post-menopausal donors (Fig. 3C). Pre-menopausal donors exhibited expression in IFITM1/2 (ST1-3, EN1-2, P/V2 and T/NK) and PLCG2 (EN1-2 and LE), which are critical for viral defense. Post-menopausal donors expressed senescence and aging markers such as ICAM1 (MP, MN, MA, and EN1-2), CXCL2 (SE, MP, MN, LE and EN1-2), ZNF31 (P/V1-2, all immune and stromal cells), ELL2 (B/P1, P/V1-2 and immune cells), and BAG3 (P/V1-2, EN1-2, SM and ST1-3)”.

7. It is challenging to comprehend why the authors present the differences in transcription factors in two ways, 3D and 3E, and why they differ. I would recommend that the authors provide additional reasoning for this choice and perhaps consider sticking with the analysis they deem superior for clarity and consistency.

We appreciate the reviewer's concern regarding the presentation of transcription factor (TF) differences in Manuscript Figures 3D and 3E and understand the need for clarity. Our intent is to provide a comprehensive view of the TF activity changes between pre- and postmenopausal cell types by presenting the data using two different but complementary analytical approaches:

The first heatmap (manuscript Fig. 3D) illustrates the absolute differences in selected transcription factor activities by cell type. This approach provides a straightforward comparison of TF activities between the two states. The second heatmap (Manuscript Fig. 3E) represents the relative changes in transcription factor activities by cell type. We included this analysis to ensure that TFs with substantial relative changes, which may not be apparent when looking at absolute differences alone due to their low baseline activities, are not overlooked. This method highlights TFs that may have a significant biological impact, even if their absolute activity changes are small.

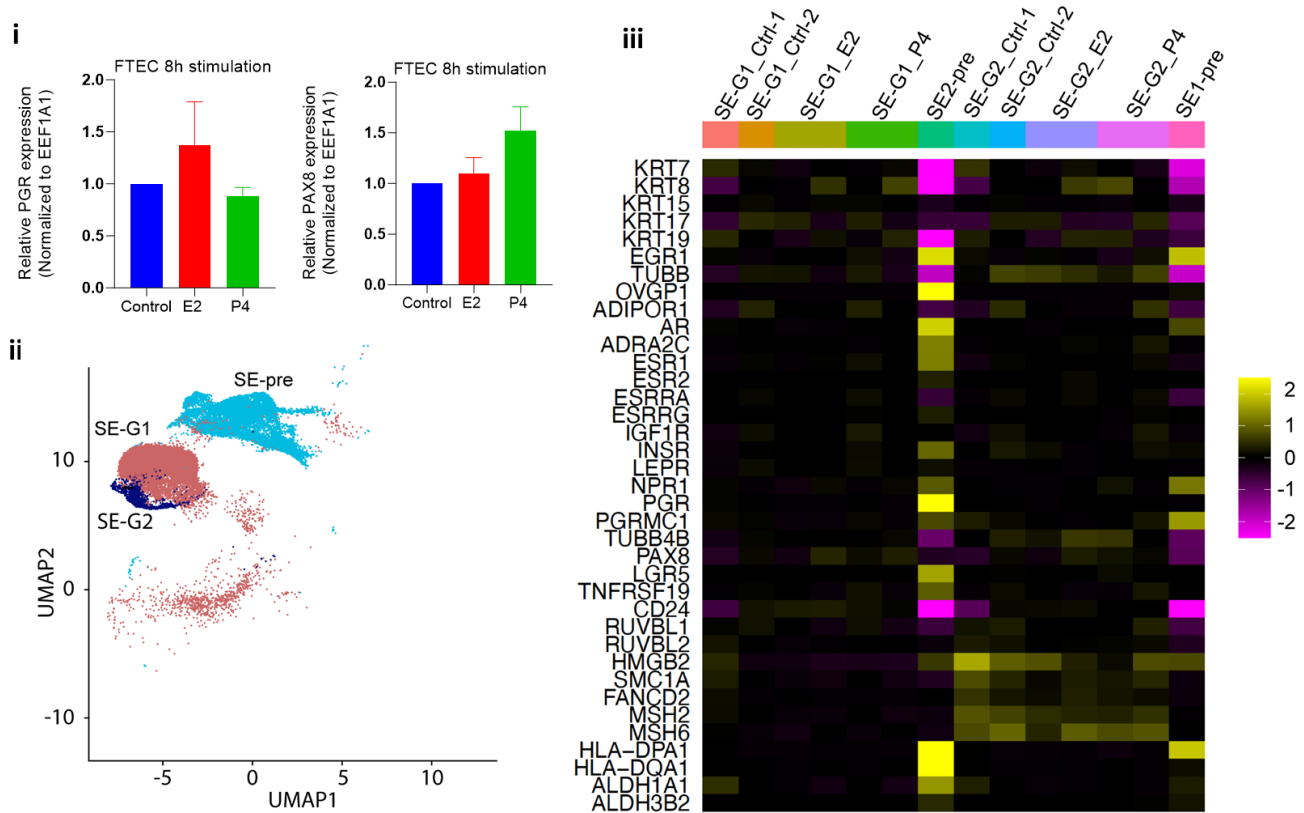
The inclusion of both analyses allows us to capture both the scope and the scale of the transcriptional shifts occurring across menopause. By providing these dual perspectives, we aim to offer a more nuanced understanding of the TF dynamics. We believe that each method offers unique insights and, together, captures a fuller picture of the cellular changes that characterize the transition into post menopause.

Secretory epithelial cells change more significantly than other cell types between pre- and post-menopausal donor samples.

Overall, the authors' findings give rise to a crucial question: how the observed transcriptomic changes manifest as functional alterations in individual cell types? The authors might explore conducting functional assessments in SE cells using FT epithelial organoids and draw comparisons between pre-menopausal and postmenopausal samples or between proliferative and secretory phases.

We tried to assess SE cell functions and molecular changes by stimulating primary FT epithelial (FTEC) *in vitro* with estrogen (E2) and progesterone (P4) and to confirm the expression of menstrual cycle-dependent genes. Using qRT-PCR, we observed the upregulation of PGR in FTEC cells treated with E2, while P4 treatment lead to the upregulation of PAX8 (**Rebuttal Fig. D-i**).

Next, scRNA-seq of FTEC stimulated with E2 and P4, was performed to assess if we can recapitulate the distinct cellular states of SE cells, we observed during the menstrual cycle phases. After integration of the *in vitro* cells with our pre-menopausal SE data, we observed that the majority of *in vitro* FTEC clustered together in SE-G1/G2 regardless of hormone stimulation (**Rebuttal Fig. D-ii**). The same was observed for stromal cells (*Data not shown*). Furthermore, we observed that *in vitro* cultured cells (SE-G1/G2) clustered distinctively separate from premenopausal SE-pre cells in the UMAP space (**Rebuttal Fig. D-ii**). Gene expression analysis of *in vitro* stimulated and pre-menopausal SE cells revealed significant differences between samples. We previously showed that OVGP1 expression in SE-pre cells was significantly different between menstrual cycle phases, but scRNA-seq of *in vitro* FTEC cells did not detect OVGP1 mRNA expression in any treatment settings (control vs E2 vs P4). OVGP1 was only detected in pre-menopausal tissue derived SE cells (**Rebuttal Fig. D-iii**). Similar results were observed for the expression of IGF1 and IGF2 in FTSC in our *in vitro* analysis (*Data not shown*). This could indicate that SE and ST cells require communication/ interaction (e.g., through secretion of ligands etc.) that leads to the upregulation of certain genes (OVGP1, IGF1 and IGF2). Furthermore, our data also



Rebuttal Figure D: Primary fallopian tube tissue cultures cannot recapitulate pre-menopausal SE cell states. i) FTEC cells were isolated and treated with estrogen (E2) and progesterone (P4) at indicated time points. RNA was isolated and analyzed to validate the induction of genes found in each menstrual cycle phase. ii) Integration of our *in vitro* (SE-G1/G2) and tissue derived SE (SE-pre) cells into the same UMAP space. iii) Heatmap showing scRNA-seq analysis of genes found in pre-menopausal SE cells (SE1/2-pre) and *in vitro* cultured and treated FTEC (SE-G1/G2) cells.

strongly indicates that *in vitro* cultured cells behave very differently and that *in vitro* systems might not be able to recapitulate the intricate dynamics occurring in tissues. Based on this finding we believe that epithelial organoids might not be sufficient either and that more advanced organoid models with stromal components may be required.

1. Based on the UMAP profiling, SE post-menopausal cells seems to be the same, so what is the reason to divide them in two subtypes? Authors should provide a clearer explanation of the dissimilarities and validate that these distinctions are not merely artifacts arising from the integration process.

To identify subtypes within postmenopausal SE cells, we applied a hierarchical clustering approach named HIPPO, which is based on UMI count data (**Figure A-i**). Subsequently, we integrated the results from the HIPPO analysis with UMAP visualizations of both pre- and post-menopausal SE cells. This analysis revealed a distinct major cluster branch, incorporating sub-clusters 2 and 10 (**Figure A-i**), which did not coincide with any clusters identified in pre-menopausal SE cells (**Fig. A-ii**). We have designated this unique cluster branch as the SE-post-specific cluster. We note that HIPPO clustering operates on zero-proportions from raw UMI data, and not integrated data. These distinctions are not artifacts from the integration process. Furthermore, this cluster shows unique expression patterns and TF activities. Moreover, p53 immunohistochemistry in post-menopausal patient D5 revealed a small subset of fallopian tube epithelial cells with strong nuclear expression of p53 (**Fig. A-iii**).

2. According to the data presented in Fig 4C, the proportion of cells expressing hormone receptors ESR1 and IGFIR is relatively consistent between pre- and post-menopausal samples. In contrast, there is a higher proportion of cells expressing ESR2 and LEPR in post-menopausal samples compared to pre-menopausal ones. Please review and modify the content of these paragraph: “In post-menopausal SE cells, the overall expression levels of hormone receptors are decreased but a small proportion of cells highly expressed ESR1, ESR2, IGFIR and LEPR”. Further clarification of these results should be included in the discussion section.

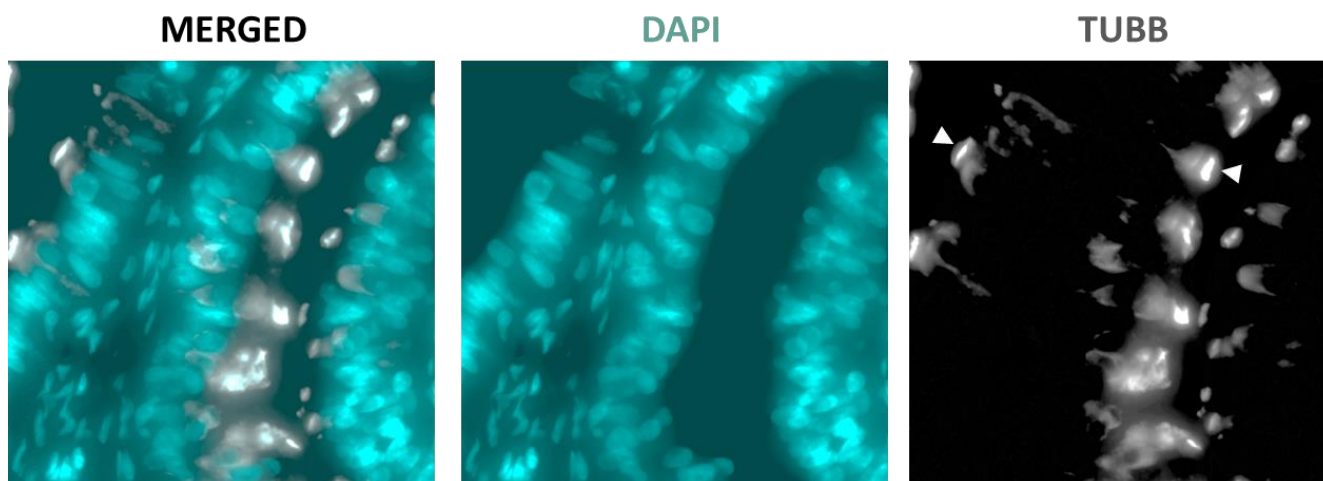
The sentence has been modified as follows: “In post-menopausal SE cells, the overall expression levels of hormone receptors are decreased but there is a small proportion of cells represented by the SE-post cluster with a higher average expression of *ESR2*, *IGF1R* and *LEPR*.” Please also refer to **Rebuttal Figure C** for patient-specific expression of ESR2, IGF1R and LEPR in SE cells.

3. What prompts the sudden examination of keratin when it hasn't been discussed in previous analyses?

In the secretory cell subtype “SE-post-specific” we observed a consistent increase of keratins (KRT15, 17, 19) and considered this finding of importance due to their structural support functions that are known to decline during aging and as possible markers of cancer cell progenitors. Hu et al., 2020 described a KRT17+ secretory cell cluster in the tubal epithelium of women with ovarian cancer (PMID: 32049047). KRT15 positive breast luminal progenitor cells were identified as the cell of origin for basal-like breast cancer. Of note, KRT15 positive luminal progenitors also overexpress KRT19 (PMID: 35821504).

Additionally intriguing is the expression of TUBB in SE3 pre. Why not bring attention to it? What is your perspective on its potential role within this particular group?

SE3-pre cells showed increased expression of genes for cell cycle, chromatin remodeling and DNA repair. TUBB is a major constituent of microtubules, essential during mitosis and in cilia. Therefore, we believe that TUBB overexpression is either an indicator of cell cycling or of transitional cilia in these cells. **Rebuttal Figure E** highlights TUBB localization to FT cilia. This was added to the text.



Rebuttal Figure E: Immunofluorescence of human fallopian tubes. Nuclei stained with Hoechst/ DAPI (Cyan), while TUBB is localized to the cilia of ciliated epithelial cells (arrow heads).

4. Concerning the TF enrichment analysis, it would be convenient to show just one figure—either depicting TF activities or z-scores—because presenting both might lead to confusion in drawing conclusions.

Our intent was to provide a comprehensive view of the TF activity changes between pre- and postmenopausal cell types by presenting the data using two different but complementary analytical approaches. The first heatmap (Fig. 3D) illustrates the absolute differences in selected transcription factor activities by cell type. This approach provides a straightforward comparison of TF activities between the two states. The second heatmap (Fig. 3E) represents the relative changes in transcription factor activities by cell type, expressed as a percentage change from the pre-menopausal levels ($((\text{Post} - \text{Pre}) / \text{Pre} * 100)$). This method highlights TFs that may have a significant biological impact, even if their absolute activity changes are small. The inclusion of both analyses allows us to capture both the scope and the scale of the transcriptional shifts occurring across menopause. By providing these dual perspectives, we aim to offer a more nuanced understanding of the TF dynamics at play. We believe that each method offers its unique insights and, together, they paint a fuller picture of the cellular changes that characterize the transition into post menopause.

5. The spatial transcriptome data in this research only capture alterations in certain genes in pre-menopausal samples. How many samples did you used? To appropriately validate the scRNA-seq findings, it is essential to incorporate spatial transcriptomics data from both pre- and post-menopausal samples to further investigate the spatial-specific changes during the menstrual cycle or during the aging process. Kindly incorporate this information, if possible.

We show one patient. We do not have spatial transcriptomics data on post-menopausal patients. Given the costs, this was prohibitive for our laboratory. As requested, we have now incorporated detailed information on the tissue sample utilized for Visium analysis, and the subsequent analysis methods into the methods section of our manuscript. Spatial transcriptomics was performed on a separate pre-menopausal patient (D19; please refer to amended table S1). We also edited the methods section.

Raw sequencing data of 10X Visium of sample D19 was pre-processed using spaceranger (v1.3) from 10X Genomics, then the data was loaded using *Load10X Spatial* function in R Seurat and normalized using *sctransform*. *SpatialFeaturePlot* function was applied to visualize the gene expression in the histology image.

6. Line 238: Regarding spatial transcriptomics, from which samples were these analyses performed? Why is this validation limited to confirming scRNAseq data only? Spatial transcriptomics offers a wealth of information, and it would be beneficial to include more details about its application and insights gained.

We amended Manuscript Table S1 to include information on the sample (patient D19).

During the menstrual cycle, secretory epithelial cells change more than other cell types

1. Statistical analysis should be conducted on the quantification of OVGP1 protein expression staining to determine the significance of differences between secretory and proliferative conditions.

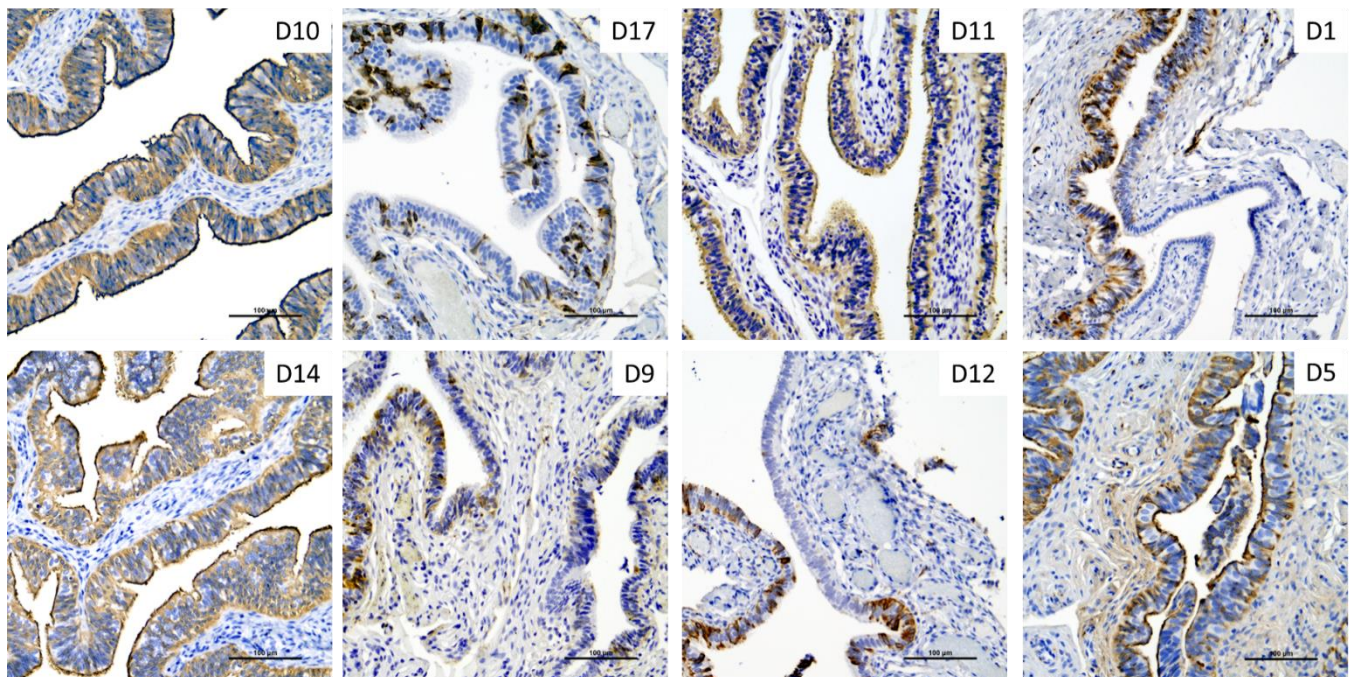
Differential expression (DE) analysis was performed to identify differentially expressed genes between secretory and proliferative conditions. OVGP1 has FDR corrected p-value of 3.1×10^{-30} and a fold change of 13.48. Our study included 3 representative FT tissues in the secretory phase and 4 patients in the proliferative menstrual cycle phase. Given the semi-quantitative nature of immunohistochemistry, it does not make sense to employ statistics on low numbers (PMID: 25432701). We revised the manuscript to refer to low or high OVGP1 expression during the proliferative and secretory menstrual cycle phases.

2. What novelty arises when subjecting a co-culture of primary human stromal and epithelial cells, obtained from patients operated on for benign conditions, to stimulation with progesterone and estrogen treatment (E2)? The association between IGF1 and IGF2 after hormonal treatment has already been documented.

This experiment was performed to understand if the scRNA results can be confirmed *in vitro*. The hormonal association between IGF1 and IGF2 have been documented in the uterus and endometrial cancer but not in the fallopian tube. Probably tubal cell-to-cell communication plays a more important role in fertility and fallopian tube signaling as shown in **Rebuttal figure D**.

3. Line 254: The images in Figure S4C seem to show a high presence of OVGP1 in one of the progesterone and one of the postmenopausal images. This observation contradicts the described result in the text, making it inappropriate to assert that SE1-pre cells are regulated by progesterone.

While we do observe OVGP1 expression in the FT epithelium of progesterone treated and in



Rebuttal Figure F: OVGP1 immunohistochemistry in the proliferative phase (patients D10 & 14), secretory phase (D17 & 9), patients on progestin-based contraception (D11 & 12), and in post-menopausal patients (D1 & 5).

postmenopausal women, protein expression is considerably lower and patchy compared to pre-menopausal women in the proliferative menstrual cycle phase (**Fig. F**). OVGP1 levels vary across postmenopausal patients, because 1) Estrogen loss is a gradual process and 2) use of hormone replacement therapy, BMI and years since menopause affect estrogen levels in postmenopausal women (PMID: 19808850) (**Rebuttal Table 2**). Furthermore, it has been well described that even after menopause, estrogens are still produced by peripheral tissues (especially adipose tissue) (PMID: 29333273, 21961713). Brodowska et al., 2021 showed that ESR1 and PR expression in the fallopian tube epithelium was observed even 11 years after the last menstrual cycle (PMID: 34501783).

In our data it seems that OVGP1 expression is correlated with hormone replacement therapy, as women who substitute estrogen or recently underwent menopause (D2, 3, 5) have higher OVGP1 expression (**Fig. F and Rebuttal Table 2**). We modified our statement to: *“SE1-pre is associated with the secretory phase of the menstrual cycle, where progesterone is high, and estrogen is low but not absent.”*

Rebuttal Table 2: Postmenopausal patients with their associated BMI, OVGP1, hormone replacement therapy and

ID	Years since MP at surgery	BMI	HRT
D1	19	27.58	Vaginal estrogen
D2	20	28.47	N
D3	6	31.48	Oral progesterone/Vaginal estrogen
D4	2	21.45	N
D5	15	22.73	Vaginal estrogen
D6	10	26.74	N
D7	10	31.31	N

year from menopause to surgery

4. Line 254: The statement that SE2-pre cells and OVGP1 expression are estrogen-regulated is made, but it should be noted that this is a suggestion based on indirect results of the analysis (FIG 5C-D and S4B) and lacks validation.

We weakened this statement, but it is known that OVGP1 is an estrogen-dependent/regulated gene (PMID: 34993641, 20130498, 15666095). This is why this was not further validated.

5. Line 283: Why RNA velocity was not performed on post-menopausal samples? Including these samples could reveal a drift from pre-menstrual to post-menstrual populations. It would also be interesting to conduct RNA velocity analysis on all cell types in premenopausal samples, not just SE.

We choose to report RNA velocity on pre-menopausal SE cells because we observed a clear cell population shift/ presence (Manuscript Fig. 5A) and the highest number of up- and down-regulated DEGs by menstrual cycle phase (Manuscript Fig. 5E), indicative of a cyclical process. While we agree it would be interesting to perform this analysis on other cell types as well, our manuscript focuses specifically on epithelial and to a lesser extent, on stromal cells. Ultimately, it was also a world limit issue, and we tried to limit our analysis to the cell types with the greatest changes and significance to disease processes.

Secretory epithelial cell markers can subtype high grade serous ovarian cancer

1. This section is the most relevant and novel of the paper. The deconvolution analysis presented in figure 6, should be the basis to prove the functional relevance of this finding using an in vitro model of FT epithelial organoids, otherwise it will remain as descriptive and speculative.

While we completely agree and believe that this is important for future/ follow up studies, it is outside the scope of this study. Nevertheless, our laboratory has started to establish FT organoid cultures as described by Kessler et al., 2015 in *Nat. Comm.* While we observe TUBB expression and observe ciliated cells in our epithelial organoid culture (**Rebuttal Figure G**).

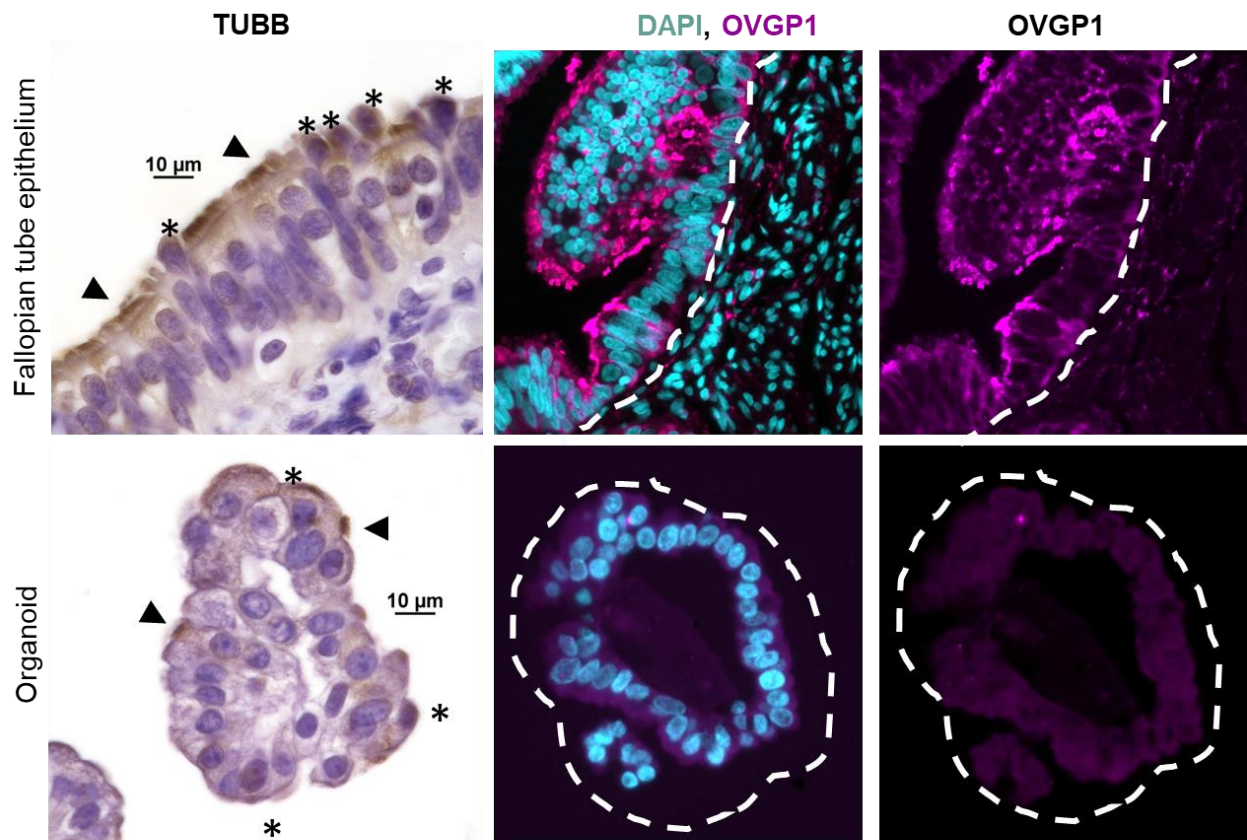


Figure G: Immunohistochemistry (IHC) of TUBB, and immunofluorescence of OVGP1 in fallopian tube epithelium and fallopian tube epithelial organoids. Arrowheads indicate the presence of ciliated fallopian tube epithelial cells (TUBB positive cilia), while stars indicate non-ciliated fallopian tube epithelial cells in IHC. Immunofluorescence of OVGP1 in human fallopian tube epithelium and epithelial organoids revealed that in vitro culture conditions without E2 are not sufficient to drive OVGP1 expression. White dashed lines/ circles indicate epithelial margins in the fallopian tube and in organoid cultures.

Furthermore, we found that Dinh et al., 2021 and Ulrich et al., 2022 reported a transitional (unclassified 2+3) and a NCSE 2-6 cell cluster respectively, that showed overlap in genes expressed with our SE3-pre cluster (**Rebuttal Fig. H**). We also found that SE3-pre was enriched in similar pathways (cell division/ mitosis and immune cell signaling/ interactions) as unclassified 2+ 3 (Dinh et al., 2021; Fig. 3D), expressed similar marker genes such as RUNX3, EPCAM, OVGP1, FOXJ1, PTPRC, CD3E, KRT10 (Dinh et al., 2021; Fig 4D/F. Ulrich et al. 2022; Fig. 3B-G) and most strikingly, we found that the transitional/ unclassified 2-3 cell cluster was enriched in genes associated with the immunoreactive molecular subtype of high-grade serous ovarian cancer (Dinh et al., 2021, Fig. 5C). We performed immunohistochemistry of the lymphoid markers CD3E and RUNX 3 (**Rebuttal Figure H**), which revealed CD3E and RUNX3 positive cells at the basement membrane of the fallopian tube epithelium. These cells displayed lymphoid characteristics as described by Ulrich et al., 2022, suggestive of basal or resident memory T cell identity. If these cells are indeed lymphoid cells with epithelial signatures or vice-versa, FT epithelial organoids may not be suitable to fully study functional relevance.

Methods

1. What is the resolution for clustering? A statistical approach to identify the resolution or find the number of clusters should be performed.

The Shared Nearest Neighbor (SNN) graph for graph-based clustering was constructed using Seurat's *FindNeighbors* and *FindClusters* functions, with an initial resolution parameter set at 0.5. This value was chosen as a starting point based on our preliminary analysis, which provided a balance

between granularity and meaningful biological interpretation. The major cell types are informed by canonical markers and visual examination of UMAP plots.

We employed the HIPPO statistical approach for further subtyping within the SE and T/NK cell populations. This hierarchical clustering analysis allowed us to explore and evaluate different numbers of clusters objectively. Through HIPPO, we generated a hierarchical clustering dendrogram and rigorously examined various cluster solutions. The final number of clusters presented in our results was determined after thorough exploration, guided by statistical robustness and biological relevance rather than an arbitrary resolution setting. This iterative process ensured that the cluster selection was not solely based on the resolution parameter but substantiated by a combination of statistical and biological evidence.

2. How were batch effects addressed when combining single-cell RNA sequencing (scRNAseq) and single-cell ATAC sequencing (scATACseq), or when merging databases from pre- and postmenopausal samples? Did the correction involve the inclusion of a design matrix in the scaling procedure, and were the influences of cell cycle genes eliminated through regression?

In addressing batch effects for the integration of scRNAseq and scATACseq data, as well as the combination of pre- and post-menopausal sample databases, we employed the RPCA method as implemented in Seurat v4.0. This approach is a refinement of the canonical correlation analysis (CCA) used in previous versions of Seurat. It projects the datasets into a subspace that enhances the identification of correlations between datasets. In this subspace, mutual nearest neighbors (MNNs) are calculated and used as 'anchors' for batch correction, aligning the datasets while preserving intrinsic biological variation. The RPCA method's efficacy in batch effect correction, especially in large datasets, has been validated in a comparative study published in *Genome Biology* (<https://genomebiology.biomedcentral.com/articles/10.1186/s13059-019-1850-9>). In this study, Seurat 3—which uses a similar approach to Seurat 4—was ranked highly effective for batch effect correction, surpassing other methods, including scGen and the earlier Seurat 2.

Regarding cell cycle genes, we considered their expression levels, which are generally lower than those of the highly expressed genes selected as 'anchors' for dataset correction. Given the robust performance of the RPCA method in our analysis and the comparative study's findings, we believe that the influence of cell cycle genes on our results is minimal. Consequently, we did not perform regression on cell cycle genes but remain confident in the trustworthiness and robustness of our integrated data.

3. The data preprocessing pipeline outlined in the Methods section, named 'scRICA,' is based on the GitHub and R Vignettes and has been developed by the authors. However, it has not been formally published to date. The manuscript should provide a detailed description of the procedures, explicitly stating whether the CellRanger suite was utilized for certain preprocessing quality controls and any subsequent implemented steps. Additionally, it is crucial to disclose the versions of packages and software used in this pipeline.

We added software version information to the methods section.

4. There is no reference to ambientRNA correction, a well-known artifact that significantly influences the downstream interpretation of results. The authors should provide evidence of this quality control (QC) step, along with details on the other QC-based filters applied. Including a table specifying the number of cells filtered at each step would enhance the transparency of the methodology.

We appreciate the reviewer's focus on correcting for ambient RNA, which is a known artifact in single-cell RNA sequencing workflows that can affect downstream analysis. To clarify, our quality control (QC) protocol carefully considers ambient RNA levels. According to guidelines provided by 10X Genomics, correction for ambient RNA is particularly crucial when certain indicators are present, such as the "Low Fraction Reads in Cells" alert or a lack of a distinct inflection point in the barcode rank plot.

During our study, neither of these warning signs were observed in our data, suggesting that the level of ambient RNA did not reach a threshold necessitating correction. However, to ensure full transparency and provide assurance of data integrity, we have included detailed information regarding our QC steps and the subsequent results in Supplementary Table 2.

5. Some results indicate variations in cellular abundances between pre- and postmenopausal women. The approach taken to assess differential abundances involves conducting a t-test comparison between the number of cells in one condition versus the cells in the other condition. However, this analysis fails to consider the topology of observations in the transcriptomic space beyond the assigned cell type tags. It is crucial to note that differential cell abundance analysis should ideally be conducted in an unsupervised manner. Cells are distributed along a continuum, and as such, there may be overlooked regions of different abundances (e.g., smaller, larger, or between clusters). To fully harness the potential of single-cell data, a more nuanced analysis is recommended. Numerous studies explore differential abundances between conditions, and there are several published methods that could enhance the analysis. I suggest considering at least one of these methods to accurately assess regions of differential abundance in the data.

Thank you for critically evaluating our approach to assessing cellular abundances between pre- and postmenopausal women. We agree that the t-test comparison for differential cell abundance does not capture the complex topology of the transcriptomic landscape. Indeed, such analysis may not consider the continuous nature of cellular distribution, potentially overlooking nuanced regions of varying abundances.

While our study did touch upon cellular abundances, we concur with the consensus in the single-cell transcriptomics field regarding the limitations of inferring cellular abundance directly from single-cell data. These limitations include the susceptibility of such data to technical and experimental artifacts, which we have been careful not to overinterpret.

Therefore, our conclusions are not predicated solely on cellular abundance. Our more substantive analyses hinge on differential gene expression, which utilizes the multidimensional information of thousands of genes to discern the nuanced differences across conditions. This approach allows for a more robust and comprehensive understanding of the underlying biology, and it is this analysis that forms the core of our findings.

6. Line 578 states, 'We summarized cell numbers based on cell annotations and normalized the relative cell percentage by the total number of cells. We only reported significance with $p < 0.05$.' Could you please clarify if this statement pertains to Cell Abundance analysis? Kindly provide an explanation and state this explicitly.

This was added to the methods section.

7. The Methods section also lacks the application of advanced tools for RNA velocity analysis. While Velocyto is a powerful tool, it is recognized that inference may be biased by unmodeled transcription kinetics (REF. Bergen V, Soldatov RA, Kharchenko PV, Theis FJ. RNA velocity-current challenges and future perspectives. Mol Syst Biol. 2021 Aug;17(8):e10282. doi: 10.15252/msb.202110282. PMID: 34435732; PMCID: PMC8388041). To enhance the reliability of their results, the authors should address this potential bias in their analysis.

Thank you for highlighting the importance of considering the limitations associated with RNA velocity analysis tools. In our study, we have employed scVelo (PMID 32747759) for RNA velocity analysis, which employs a likelihood-based dynamical model to account for full transcriptional dynamics, including splicing kinetics. This method represents an advancement over Velocyto by providing a more comprehensive model of transcription kinetics, which can reduce bias in velocity inference. We believe that scVelo's approach mitigates the potential biases that were a concern with earlier tools, thus enhancing the reliability of our RNA velocity results.

8. Furthermore, the analysis lacks some discussion and graphical support for the gene expression of features that drive the overall kinetics and the patterns supporting the obtained trajectory. Incorporating this information could offer crucial biological insights and provide additional support for the presented results.

Regarding the gene expression features and their influence on the kinetic patterns and trajectories observed, we acknowledge the potential value of this suggestion. We now performed an extensive velocity trajectory analysis using scVelo, the results did not yield additional biological insights. Thus, we chose not to include these findings in the manuscript.

9. The integrative analysis of scRNAseq and scATACseq was conducted using transfer anchors (refer to the pipeline for details) to annotate cell types. Cells with a prediction score greater than 0.5 were retained. Could the authors provide an explanation for the choice of this cutoff, as opposed to, for instance, 0.7, which might yield more reliable results?

We adopted a threshold of 0.5 for retaining annotated cells based on guidelines from the Seurat vignettes on 'Integrating scRNA-seq and scATAC-seq data.' These vignettes indicate that cells with prediction scores below 50% are likely to be incorrectly annotated, which informed our decision to apply a 0.5 cutoff. This threshold serves to exclude cells with a high probability of misannotation while retaining a robust dataset for downstream analysis. While a higher cutoff, such as 0.7, could potentially improve the reliability of cell type annotations, it also runs the risk of excluding correctly annotated cells, especially those with moderate prediction scores that still represent meaningful biological signals.

Discussion

- In the discussion (lines 323-334), the authors discuss the presence of a "SOX9+, LGR5+" population. If these markers are considered relevant, they should be introduced earlier in the text, which is not currently done. Additionally, the SE2 population appears to be substantial for a somatic stem cell population, and the authors do not provide references justifying the existence of such a population in the fallopian tubes.

We made the following edits to the manuscript: *"The SOX family, especially SOX-9/13, involved in tissue repair and regeneration, showed activities in all pre-menopausal SE groups, but with highest activity in SE2-pre indicating a higher regenerative potential of SE2-pre."*

Previous research by Paik et al., 2012 showed the existence of stem-like epithelial cells (EPCAM+, CD44+, ITGA6+) in the FT (PMID: 22911892). In 2014, Ng et al., showed that LGR5 marks stem/progenitor cells in ovary and tubal epithelia (PMID: 24997521).

-Line 330: In the endometrium, SOX9+ and LGR5+ cells have been proposed as stem/progenitor cells, representing a small fraction within the analyzed epithelial cells in the corresponding study. However, in this case, SE2 cells constitute 84% of SE cells in the pre-menopausal sample. What could explain such a large stem population in the fallopian tube?

Garcia-Alonso et al. 2021, showed that LGR5+ SOX9+ epithelial cells are enriched in the luminal epithelium of the human endometrium and are enriched during the proliferative phase of the menstrual cycle. In their paper, SOX9+ LGR5+ comprised 1150 of the 3488 cells assigned to the proliferative phase. We rephrased the discussion as follows:

"In the endometrium this menstrual cycle phase is characterized by SOX9+ LGR5+ epithelial cells. SOX9 and LGR5 are expressed in stem cells/ progenitor cells. Given that the uterine and FT epithelium share the mesoderm as a common embryological origin, we speculate that the SE2 cells in the FT are specialized, highly proliferative cells responsive to estrogen."

- SOX9 is not mentioned in the results, and it is the only gene (along with LGR5) that appears in the discussion of healthy populations with no relation to cancer.

We now included SOX9 in the results section. Please also refer to our previous answers.

-Have the authors examined the expression of SOX9 and LGR5 genes in epithelial cells throughout their visium experiments to assess whether the populations spatially behave as previously described for the endometrium (as referenced in this paper)?

The endometrium is architecturally very different compared to the fallopian tube epithelium. During the proliferative phase the endometrium undergoes significant changes and forms pronounced luminal epithelium (LE) and glandular epithelium (GE). Both epithelia differ in their development, spatial location, and biologic functions (PMID: 25023676), whereas the fallopian tube epithelium does not undergo menstrual cycle specific gross architectural (i.e. spatial) changes.

- In general, the manuscript frequently mentions genes and factors, either for defining populations or due to differential expression. However, the authors should provide more comprehensive explanations of the biological implications of these genes to enhance the understanding of fallopian tube functioning, accompanied by relevant references.

We included additional explanations and references, but we are limited by word counts/restrictions set by the journal and editors.

- I recommend a more thorough exploration of the results in the discussion section. A detailed analysis and interpretation of the findings would significantly enhance the overall comprehension of your research. Additionally, consider incorporating references that support and strengthen your results.

We have rewritten the discussion section but had to respect space constraints from Nature Communications.

Minor comments

Table S1. What is the meaning of N/A In relation with the TF enrichment analysis, it would be convenient to show only one figure (either TF activities or z-score) since the conclusion could be confused? It denotes either not applicable or not available. Please clarify.

This comment is confusing. In Table S1 N/A denotes not applicable under the menopausal age for the pre-menopausal patients. We explained why we show TF activities using two different heatmaps in the previous comments above.

Figure 1A and Figure S4A both depict the uterus throughout the menstrual cycle with the same content. It is advisable to retain one of these figures and eliminate the redundancy.

We removed figure S4A in the revised version of the manuscript.

Figure S2.B: Some gene symbols are overlapping, making it difficult to read them.

We edited Fig. S2B to have gene symbols in black font and dispersed overlapping symbols.

Figure S3.D: Heatmap titles are at different heights. Try to plot them more uniformly.

This has been addressed.

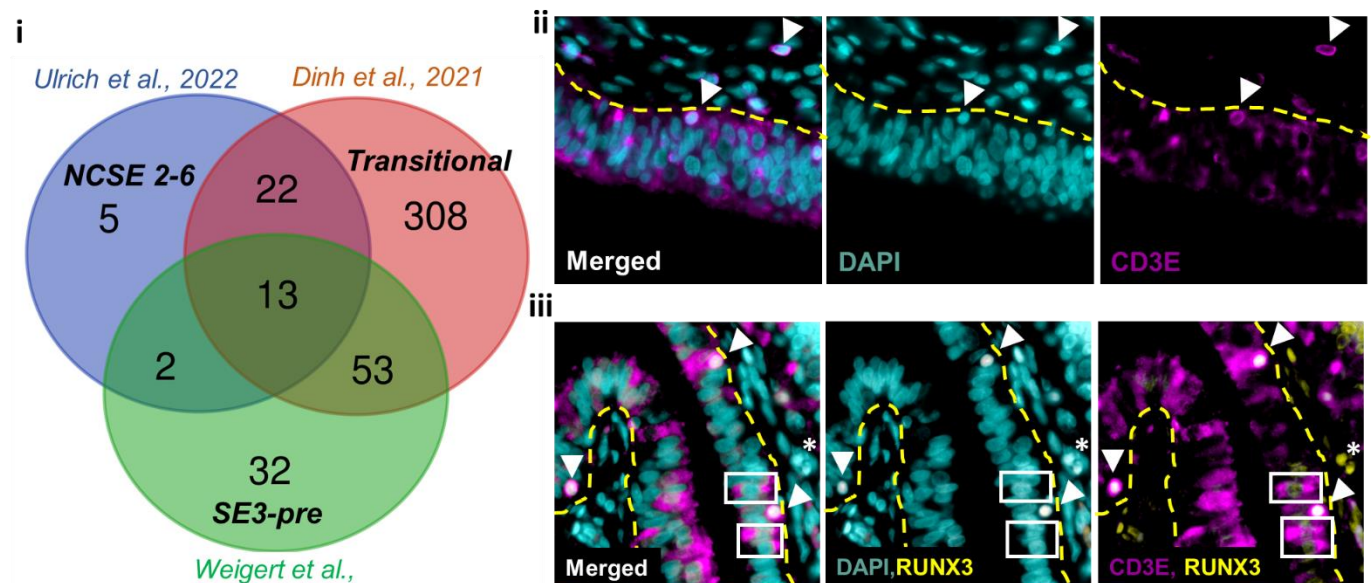
Figure 2.D.: Same as S3.D.

This has been corrected.

Fig 4A: Are 58 cells enough to determine a cell cluster???

The final number of clusters presented in our results was determined after thorough exploration, guided by statistical robustness and biological relevance. SE3-pre has quite unique cell cycle gene expression patterns. We know there are large variations between samples, as expected with patient heterogeneity, but think this small cluster of cells may contain interesting biological findings. Furthermore, we found that Dinh et al., 2021 and Ulrich et al., 2022 reported a transitional (unclassified 2-3) and a NCSE 2-6 cell cluster, that showed overlap in overexpressed genes with our SE3-pre cluster (**Rebuttal Fig. H-i**). We also found that SE3-pre was enriched in similar pathways (cell division/ mitosis and immune cell signaling/ interactions) as unclassified 2 and 3 (Dinh et al., 2021; Fig. 3D), expressed similar genes

such as RUNX3, EPCAM, OVGP1, FOXJ1, PTPRC, CD3E, KRT10 (Dinh et al., 2021; Fig 4D/F. Ulrich et al. 2022; Fig. 3B-G, **Rebuttal Fig. H-ii-iii**) and most strikingly, we found that the transitional/ unclassified 2-3 cell cluster was enriched in genes associated with the immunoreactive molecular subtype of high-



Rebuttal Figure H: i) Venn diagram showing overlap for overexpressed genes between our SE3-pre, NCSE 2-6 and transitional/ unclassified 2-3 cell clusters previously identified. ii) CD3E (lymphoid marker) immunofluorescence in human fallopian tubes reveals CD3E positive cells immune cells in the fallopian tube stroma and at the base membrane (yellow dashed line) of the FT epithelium (white arrows). iii) Immunofluorescence of the lymphoid markers RUNX3 and CD3E in human fallopian tubes. Arrows indicate RUNX3 and CD3E positive cells at the fallopian tube base membrane with prominent spherical nuclei and scant cytoplasm, indicative of basal or resident memory T cells. White stars indicate RUNX3 positive cells in the underlying fallopian tube stroma. White boxes indicate RUNX3 and CD3E positive cells in the fallopian tube epithelium that have a classical simple columnar shape (tall narrow cells with an oval nucleus close to the basement membrane).

grade serous ovarian cancer (Dinh et al., 2021, Fig 5C). Ulrich et al., 2022 showed that NSCE2-6 expressed lymphoid cell markers including CD3E, RUNX3 and PTPRC. We believe that the herein described cell clusters (SE3-pre, NCSE 2-6 and Transitional/ unclassified 2-3) may represent a similar cells type/ cluster or even the same cell type.

Figure 4.D.: Same as S3.D and 2.D.

[This has been corrected.](#)

Fig 5E, 5C, 5D. To enhance the reviewer's understanding and facilitate future readers' comprehension, it is recommended that the sequence of figures aligns consistently with the corresponding explanations throughout the manuscript.

[This has been corrected.](#)

Figure 6.A-B.: Same as S3.D, 2.D, and 4.D. "High-grade serous Ovarian Cancer" is the title of 6B heatmap. Please include a descriptive title.

[This has been addressed.](#)

Line 116: replace "Table 4" for "Table S4"

[Done. Please note that the previous Table S4 is now Table S5.](#)

Lines 163 and 164: Why are those markers associated with each function? Could the authors provide references supporting these associations?

We included references as requested.

Please indicate the versions of the software used to perform analyses: Line 485 “scRICA”, Line 488 “DoubletDecon”, Line 494 “HIPPO”, and Line 515 “clusterProfiler”.

We have added the version numbers.

Please specify the specific software used to perform the integration of scRNAseq and ATACseq datasets.

We expanded the methods section on the integration of scRNA-seq and scATAC-seq datasets as follows: *“We used cells from matched scRNA-seq as a reference to predict and obtain cell types in our scATAC-seq data (PMID: 36543131). The variable features of the scRNA-seq data were used as a reference, while the gene activity matrix generated using Seurat’s GeneActivity from scATAC-Seq data was used as the query data. Transfer anchors were learned in Seurat using FindTransferAnchors, and cell type labels were predicted with TransferData with the scATAC-seq LSI reduction as weight.reduction input. We assigned each cell in the scATAC-seq with a cell type (subcluster) identity from the matching scRNA-seq data based on the first 30 latent semantic indexing (LSI) components corrected by Harmony, excluding the first one. We only retained cells with a prediction score > 0.5 (denoted by prediction.score.max) for further analysis. We performed label transfer for each patient sample separately.”*

Regarding the single-cell analysis packages, some are in italics (DoubletDecon at Line 488), and the others are not (scRICA at Line 485, HIPPO at Lines 494 and 496, ...). Try to standardize this.

This has been corrected in the revised version of the manuscript.

There are unnumbered references in methods. Please pass all references to the corresponding numbers.”

This has been corrected in the revision.

Reviewer #3 (Remarks to the Author):

The Fallopian tubes are the major site of origin of ovarian cancer, which had motivated an increasing number of studies to understand the physiology of this tissue and the mechanisms triggering the development of ovarian cancer. In this paper, Weigert et al look at changes in expression pattern that occur with menopause, as well as during the menstrual cycle. To achieve this, the authors performed single cell transcriptomics and single cell chromatin accessibility profiling. This study can help to understand changes occurring in healthy women during their lifetime and insights in the different events occurring during cancer development, including early event occurring before menopause but potentially rendering the tissue more susceptible to neoplastic transformation later in life.

This work is at great interest for the ovarian cancer community as well as other scientists working on the Fallopian tubes. This study shows strong evidence of the results claimed, reinforced by additional experiment like spatial transcriptomic and IHC. Results are well presented, and legends are clear enough to understand the results showed. Authors could revise the following point to a better understanding and increase the robustness of their results:

- SE post specific cluster: It is difficult to understand how this cluster has been identified. The markers of secretory epithelial cells mentioned in the table S3 doesn’t seems to be expressed on this specific cluster. Additional details on markers used to classify these cells as secretory will be beneficial. Indicating the number of cells in this specific cluster for each sample could also help to have a better view of the robustness of the analysis.

The number of cells is stated in Manuscript Fig. 4A. This cluster branch comprised a total of 392 cells, distinguishing itself by the expression of several markers not typically associated with the remaining postmenopausal SE cells aligning with pre-menopausal cells. These markers include epithelial cell markers such as KRT5 and KRT18, and progesterone-regulated genes like TUBB4B, ITGA6, and CD44,

all visualized in Manuscript Figure S3B. Additionally, we observed the expression of ESRRA and INSR, as shown in Manuscript Figure S3C, which are known to play roles in uterine epithelial lineage specification and homeostasis (PMID: 37622003). These marker expressions, combined with our clustering analysis, substantiate the classification of this group as the SE-post-specific cluster.

To define subtypes within the postmenopausal secretory epithelial (SE) cells, we utilized the HIPPO hierarchical clustering method (**Rebuttal Figure A**), leveraging UMI count data as illustrated in Figure 1A. The HIPPO analysis outcomes were then superimposed on UMAP plots of combined pre- and post-menopausal SE cells, uncovering a distinct cluster branch that includes sub-clusters 2 and 10 (**Rebuttal Figure A-i**). Notably, this cluster branch was unique to postmenopausal SE cells and did not correspond with any clusters found within pre-menopausal SE cells (**Fig. A-ii**).

- Menstrual cycle phase analysis: the number of samples per group is small, limiting the power to detect associations. Therefore, while difficult to do, you could consider increasing the number samples to increase the power of the analysis.

We agree with the reviewer that increasing sample numbers is desirable and increases the power of our analysis but believe that it is impossible to achieve this within a reasonable time frame. Since we are collecting fresh specimens from surgery, acquiring all the pre-menopausal samples described in this manuscript took us over one year. It is difficult to pre-screen women based on their menstrual cycle phase for specimen collection.

Minor :

- Fig3A: you mention 11 women for premenopausal group while you have 10 patients

This error has been corrected in the revised manuscript version.

- line 119: "We also separated EN cells....based on expression pattern of KDR and CLDN5" this is confusing, clusters are made by the unsupervised clustering analysis and the expression pattern in these sub-clusters is different.

We changed the wording in the revised manuscript as follows: "*We further subtyped EN cells into EN1 and EN2 based on the expression patterns of KDR and CLDN5, respectively.*"

- Fig3A and 5A: For this type of proportion comparison, it could be very beneficial to add the individual value on top of the box plot. This could help to see any samples strongly divergent or detect the presence of different groups of samples. Overall adding this information increase the confidence in the analysis.

We attempted to label the Figures as suggested, but ultimately decided against it because it made them very busy and with the reduction in size during printing almost illegible.

The supplementary table 'S3_rna_cellular_compositions.xlsx' includes the requested information.

- SE3 cluster: you say that there are only 58 cells Min this cluster but then perform a differential expression analysis on these cells. This analysis is very difficult to trust especially because more than half of these cells come from 2 samples. You should either provide more detail, especially on QC on these cells or remove the analysis of this cluster.

We employed the HIPPO statistical approach for subtyping within the SE cell populations. Through HIPPO, we generated a hierarchical clustering dendrogram and rigorously examined various cluster solutions. The final number of clusters presented in our results was determined after thorough exploration, guided by statistical robustness and biological relevance rather than using an arbitrary cut-off. This iterative process ensured that our cluster selection was substantiated by a combination of statistical and biological evidence. SE3-pre shows unique cell cycle gene expression patterns. We do realize that there are large variations between samples, as expected with patient heterogeneity, but we do think this small cluster of cells may contain interesting biological findings.

Additionally, we found significant overlap of the SE3-pre cluster with clusters reported by Ulrich et al. and Dinh et al. Please refer also to questions by Reviewer #2 and associated **Rebuttal Figure E**.

- Confounding effect: It is recognized that having a cohort with samples coming from very similar patient is challenging. However, in this paper the two main group differ largely in term of race as 6/7 patient of postmenopausal are white while 8/10 of the premenopausal group are African American. Although it's not clear how the race could affect the expression pattern of the Fallopian tubes, we can't exclude an impact of this on the results of this study. Therefore, this limitation should be discussed.

As recommended by the reviewer, we included this in the revised version of the manuscript in the limitations of the study section: "Ethnicity might play a confounding effect in our study since six out of seven patients in the postmenopausal cohort were Caucasian and eight out of ten were African American in the premenopausal cohort."

- Fig5C: It is difficult to understand why you compared the secretory and proliferative phase by using two different clusters (Secretory SE1 vs proliferative SE2). The results should explain more clearly this choice and the impact of the analysis.

The secretory and proliferative phases were compared using two distinct cell clusters, SE1- and SE2-pre (Manuscript Fig. 5C). This stems from our observation (Figure S4B) that cell cluster SE1-pre is solely present during the secretory phase of the menstrual cycle, whereas SE2-pre is unique to the proliferative phase (Table 3, below). The cycle-specific expression of SE1- and SE2-pre gave us a unique opportunity to analyze differential gene expression in secretory epithelial cells by menstrual cycle phase.

The goal was to understand the dynamic changes in gene expression between two phases of the menstrual cycle, which are characterized by significant hormonal and functional shifts. Focusing on SE1-pre from the secretory and SE2-pre from the proliferative phase, allowed us to directly compare the transcriptional profiles of SE cells by phase, revealing phase-specific biological processes and pathways.

Cluster	CellNo	Proliferative		Secretory	
		Total	%	Total	%
SE1	1,619	9	0.555	1610	99.44%
SE2	7,409	6,855	92.52	554	7.48%

Rebuttal Table 3: Secretory epithelial cell numbers and percentage by menstrual cycle phase and SE type.

- Sequencing information: In this paper new data are integrated with old data. While this is totally acceptable, more information on the QC of each sample should be provided to determine if the two datasets are comparable or not. This information can be added to a supplement figure and or table. Adding others relevant information about the sequencing might be considered, especially because 2 different sequencers and different number of cycles has been used for this paper.

The sequencing and QC information was added to a new Supplementary Table 2.

- Visium: This experiment is useful to increase the confidence in the results. However, this section lacks key experimental detail on how many samples (and which ones) has been analyzed by this spatial transcriptomic analysis.

We have now incorporated detailed information on the tissue sample utilized for Visium analysis, and the subsequent analysis methods into the methods section of our manuscript.

- Code: In study including computational analyses the code should be provide as a method (github link) to allow the reproduction of the results

Since this manuscript did not report any original code, we wrote in our data and code availability statement:

"This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contacts upon request."

We included the GitHub link to the software package scRICA that can be used to reproduce all the analysis in this manuscript as a reference:

“Li Y. scRICA: single-cell RNA-Seq Integrative Comparative Analysis,
Github: <https://github.com/yan-cui/scRICA/?tab=readme-ov-file#readme>; 2021.”

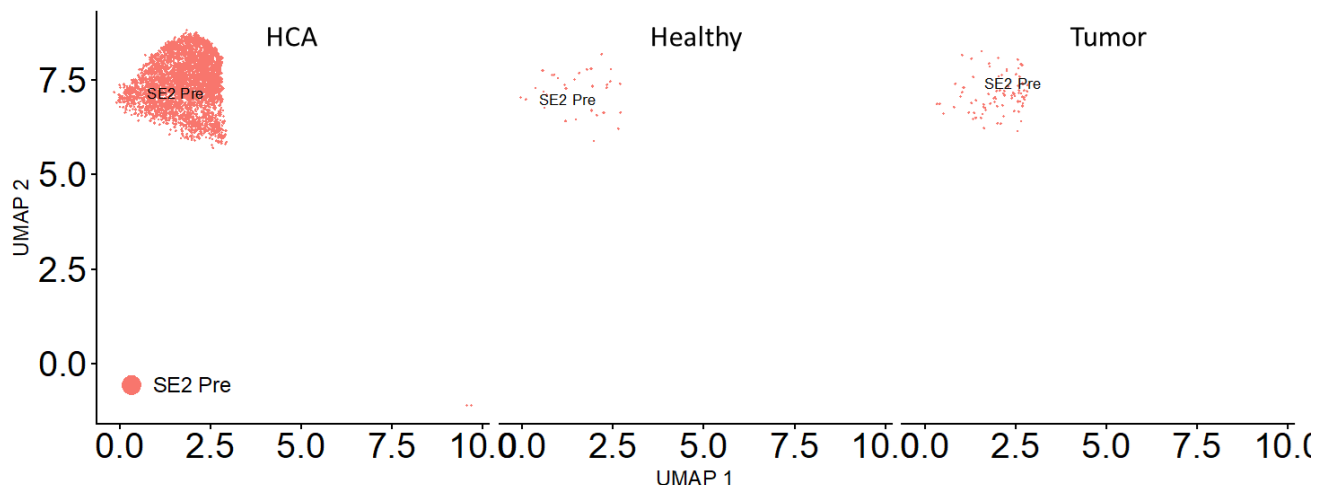
Reviewer #4 (Remarks to the Author):

This is an impressive and important manuscript that catalogues the single cell transcriptomes of the fallopian tube in premenopausal and postmenopausal women. The findings are important and complement previous reports from the authors themselves as well as previously published single cell data sets on the fallopian tube, the most likely tissue of origin of serous ovarian cancer.

My minor concern relates to figure 6. I found it hard to understand the findings and relevance of figure 6A. No quantification of the degree of overlap between the single cell data and the TCGA data in multiple tumours. Not sure what is the relevance of comparing the data with breast cancer data for example. I suggest either removing this all together or moving it to supplementary. Also, figure 6B and 6C are interesting but the classification of ovarian tumours presented by TCGA is interesting. However, a more recent classification (PMID: 32049047) with robust prognostic significance based on FT single cell sequencing would be highly relevant to this work. It would be great if this classification was considered instead of or along with the TCGA classification in figures 6B and 6C.

Regarding your insightful suggestion to incorporate the more recent ovarian tumor classification from PMID: 32049047 (Hu et al Cancer Cell 2020), we have taken steps to explore this dataset and its potential integration with our data. Despite our efforts, we found that the cell counts from their dataset are substantially lower than those in our study, with only 3,877 and 1,857 cells available for analysis. This discrepancy presents a challenge for meaningful integration, especially given the predominance of stromal (ST) cells and the limited number of epithelial cells in the dataset. Among the epithelial cells identified, the majority were categorized as CE cells, with a notable but not overwhelming presence of SE2-pre cells within tumor samples.

Given these observations and the constraints posed by the small sample size and low cell counts, we have opted not to include this analysis in our manuscript (**Rebuttal Table 4 and Fig. I**, below). We believe that doing so could risk overinterpretation of the data. Nonetheless, we acknowledge the importance and relevance of the newer classification system for ovarian tumors and its potential implications for our work. We remain committed to exploring this avenue in future research, where larger and more comprehensive datasets may allow for a more robust integration and analysis.



Rebuttal Figure I: Comparison of our dataset with Hu et al., 2020 after integration into the same UMAP space. The UMAP shows the SE2 pre in the UMAP space of human cell atlas (HCA; *our data*), the healthy and tumor cells described by Hu et al., 2020. It shows that the Hu et al dataset has much fewer cells.

clusters	cellNo	cellNo_HCA	cellNo_Healt_Per	cellNo_Tumor	cellNo_HCA	cellNo_Healt_Per_Per	cellNo_Tumor_P
BP1	1494	1494	0	0	1.14	0	0
CD4/8	18324	18226	63	35	13.89	1.62	1.88
CDB/SE3 post	66	66	0	0	0.05	0	0
CE	13297	12345	431	521	9.41	11.12	28.06
EN1	5944	5918	17	9	4.51	0.44	0.48
LE	3332	3320	8	4	2.53	0.21	0.22
MA	1010	1006	3	1	0.77	0.08	0.05
MN/MP	1782	1776	0	6	1.35	0	0.32
MP	5917	5827	8	82	4.44	0.21	4.42
P/V1	9475	9462	12	1	7.21	0.31	0.05
P/V2	114	114	0	0	0.09	0	0
SE Post Specific	497	488	9	0	0.37	0.23	0
SE Post/Pre 12	6141	5753	258	130	4.38	6.65	7
SE Post/SE2 Pre	732	710	16	6	0.54	0.41	0.32
SE2 Pre(12)	4117	4003	31	83	3.05	0.8	4.47
SE2 Pre(15)	1878	1821	43	14	1.39	1.11	0.75
SM	5415	5409	6	0	4.12	0.15	0
ST1	42239	42215	23	1	32.17	0.59	0.05
ST2	14309	10396	2949	964	7.92	76.06	51.91
T cells	869	869	0	0	0.66	0	0
Total	136952	131218	3877	1857			

Rebuttal Table 4: Total and relative (percentage) cell numbers of our data set (HCA) and Hu et al., 2020.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have responded to my comments in a thorough and comprehensive fashion. This is an important report yielding critical insights into normal physiology and homeostasis in an understudied organ.

Apologies for the delay submitting this report.

Reviewer #2 (Remarks to the Author):

Authors have made considerable efforts in the revised manuscript to address the concerns raised by the reviewers. There is a significant enhancement in the overall quality of the paper, reinforced by employing additional methodologies such as immunohistochemistry (IHC), and in vitro co-cultures. The study is well-structured, featuring clear figures and comprehensive supplementary data. However, some additional comments and concerns could be considered by authors to enhance clarity or delve deeper into certain aspects of the research.

Results

1. Cohort description and Subject Details

a. Since there are premenopausal women who have a history of gynecological infections (D10, D14-D16), and acknowledging that prior infections can influence the features and function of the fallopian tubes, can you demonstrate whether the cellular composition and their transcriptomic profile of those with and without previous infections (D11-D13, D17-D19) is comparable?

b. While there is some uncertainty and limitation about deriving reliable conclusions from performing Visium experiments with only one patient (D19), reviewer acknowledges the expensive nature of spatial transcriptomics (ST) technology. However, the ST data in this study only capture changes in specific genes and pathways analysed through single-cell RNA-seq. It would be advantageous to broaden the scope of results to investigate whether patterns of different cell

cluster localizations could be identified prior to menopause. Such observations could potentially be linked to the documented alterations in cell-cell communication.

2. Human pre-menopausal and post-menopausal fallopian tubes differ in cellular composition and gene expression.

a. The observation of reduced cell-to-cell communications with aging is fascinating. Does this happen in other organs too? While the research primarily examines the human fallopian tube, it would be valuable to extend these observations to other reproductive tissues such as the endometrium, myometrium, ovary, and/or cervix. This expansion could provide insights into the implications of menstruation cessation on immune and stromal/epithelial interactions and what are the potential biological consequences might this have, particularly regarding immune tolerance? Kindly include references to substantiate these assertions.

3. Secretory epithelial cells change more significantly than other cell types between pre- and post-menopausal donor samples.

a. What is the rationale behind researchers opting to stimulate primary fallopian tube epithelial (FTEC) cells in vitro with estrogen (E2) and progesterone (P4), rather than utilizing FT epithelial organoids to assess the functionality of secretory epithelial cells (SE cells)? As suggested, the in vitro systems developed from primary cells might not accurately mirror the complex dynamics observed in tissues. Given this limitation, and acknowledging the authors' hard work, one might argue that using epithelial organoids could have been a preferable option, as previously demonstrated by Kessler et al. in Nat Comm. 2015 or Chang et al. in J Biomed Sci. 2020. This approach would not only enable comparisons between proliferative and secretory phases but also between pre-menopausal and postmenopausal samples, a comparison lacking in this section of the reviewed manuscript.

4. During the menstrual cycle, secretory epithelial cells change more than other cell types

a. Although it's established that OVGP1 is a gene regulated by estrogen, the assertion regarding the estrogen regulation of SE2-pre cells and OVGP1 expression, as indicated by FIG 5C-D and S4B, requires referencing to reinforce its validity. It's advised to include the references recommended in the rebuttal letter to enhance the support for this statement.

Methods

1. While it's accurate that the authors have included a section dedicated to RNA velocity analysis, the details outlined in the rebuttal letter contradict those presented in the manuscript. Please, assess this section to ascertain whether Velocityto or scVelo was utilized, and include the relevant references accordingly.

2. Given that you've conducted a thorough velocity trajectory analysis utilizing scVelo, it would be greatly appreciated if you could include, at minimum in the rebuttal letter, the resultant outcomes. This would help confirm whether the impact of gene expression features on kinetic patterns and trajectories has provided any further biological insights.

Discussion

1. Kindly incorporate the reference PMID: 24997521 to support the expression/activity of epithelial progenitor genes like LGR5.

2. Although the endometrium exhibits distinct architectural characteristics from the fallopian tube epithelium, with the latter not undergoing specific gross architectural changes during the menstrual cycle, it would be intriguing to investigate the expression of SOX9 and LGR5 genes in fallopian tube epithelial cells throughout their visium experiments. This examination would enable the assessment of their spatial distribution within the fallopian tube.

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Overall this paper provides strong evidence and is of high importance for the ovarian cancer and Fallopian tube research fields.

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In spite of the above, I believe that the work is highly valuable and would enrich our understanding of fallopian tube epithelial cell biology in the context of health and disease.

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We thank the reviewer for their kind feedback.

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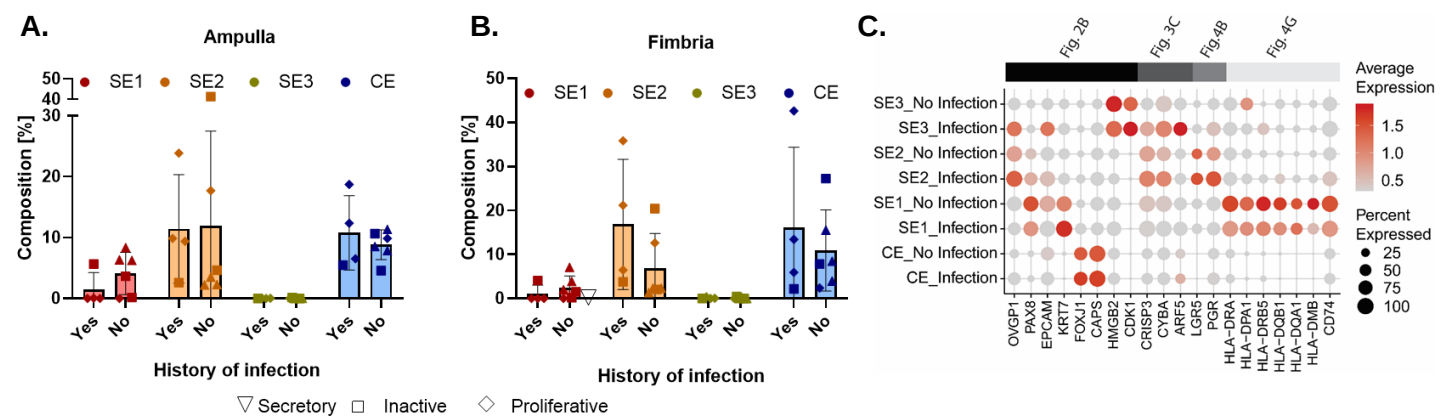
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1.Cohort description and Subject Details

a. Since there are premenopausal women who have a history of gynecological infections (D10, D14-D16), and acknowledging that prior infections can influence the features and function of the fallopian tubes, can you demonstrate whether the cellular composition and their transcriptomic profile of those with and without previous infections (D11-D13, D17-D19) is comparable?

All fallopian tubes included in this study were reviewed by a board-certified gynecologic pathologist (R.L.). We did not observe differences in the cellular composition of fallopian tube epithelial cells based on the history of infections (Rebuttal Fig. 1A-B).

Most pre-menopausal women with gynecologic infections had a history of HPV. These HPV infections had occurred +10 years before surgery. All follow-up PAP-smears in the past 5 years were HPV-negative and did not find any malignancies. While we found these patients to have a history of HPV recorded on their medical history, we cannot confidently rule out that other patients did not have a history of HPV. To address the reviewer's concerns, we focused on fallopian tube epithelial cell clusters SE1-3 and CE based on the history of infection (Rebuttal Fig. 1C). There are no significant differences in the expression of key genes identified in our study based on the history of infection in SE1,2 and CE cells. We observed slight differences in OVGP1 and EPCAM expression in SE3-pre

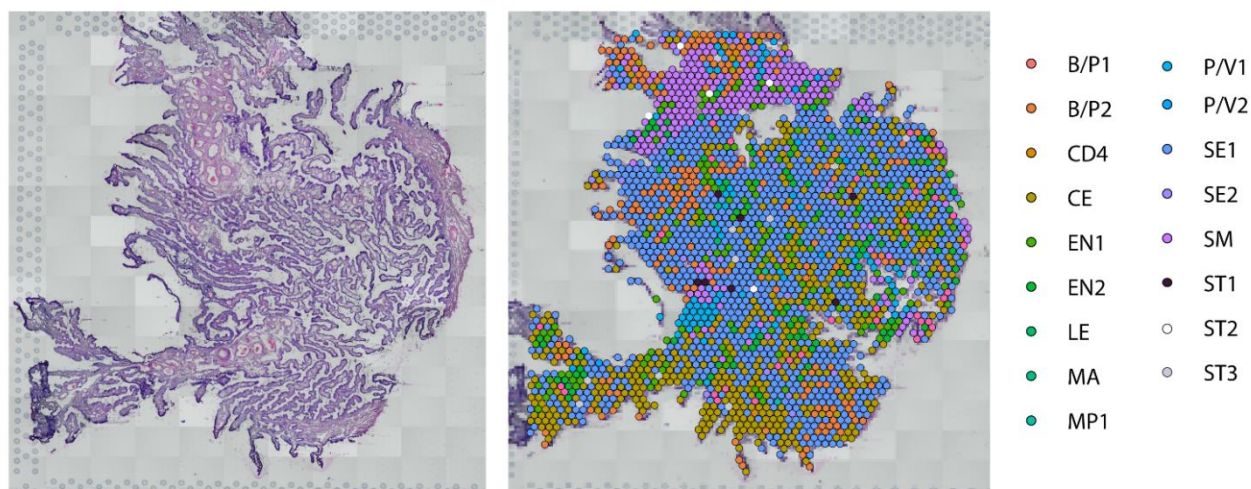


Rebuttal Figure 1: Cellular composition of fallopian tube epithelial cells in the ampulla (A) and fimbria (B) based on history of gynecologic infections. (C) Gene expression in fallopian tube epithelial cells SE1-3 and CE, in genes described in manuscript Figures 2B, 3C, 4B, and 4G. SE: secretory epithelial cells, CE: ciliated epithelial cells.

cells but believe these are due to patient heterogeneity and low cell counts (58 cells). We added an additional note to our Table S1 indicating that previous infections were HPV infections.

b. While there is some uncertainty and limitation about deriving reliable conclusions from performing Visium experiments with only one patient (D19), reviewer acknowledges the expensive nature of spatial transcriptomics (ST) technology. However, the ST data in this study only capture changes in specific genes and pathways analysed through single-cell RNA-seq. It would be advantageous to broaden the scope of results to investigate whether patterns of different cell cluster localizations could be identified prior to menopause. Such observations could potentially be linked to the documented alterations in cell-cell communication.

*The focus of our manuscript is on epithelial cells. Nevertheless, we included additional Visium analysis of existing data showing cell cluster localizations (**Rebuttal Fig. 2**). Based on the H&E from the fallopian tube fimbria, the stromal proportions are very low and therefore only few stromal cell clusters were identified in the Visium, which is also further limited by the Visium's resolution. This does not allow for meaningful statistical analysis of stromal cell cluster locations and cell-cell communication.*



Rebuttal Figure 2: Spatial transcriptomics profiling of normal pre-menopausal fallopian tube fimbria using 10x genomics Visium. Pre-menopausal fallopian tube fimbria was analyzed for the spatial distribution of major cell-type clusters. Abbreviations: SE1-2 = 2 subtypes of secretory epithelial cells; CE = ciliated epithelial cells; MP = macrophages; MA = mast cells; ST1-3 = 3 subtypes of stromal cells; SM = smooth muscle cells; LE = lymphatic endothelial cells; EN1-2 = 2 subtypes of endothelial cells; P/V1-2 = 2 subtypes of pericytes and vascular smooth muscle cells; B/P1-2 = 2 subtypes of B cells and plasma B cells, CD4= lymphocytes.

2.Human pre-menopausal and post-menopausal fallopian tubes differ in cellular composition and gene expression.

a. The observation of reduced cell-to-cell communications with aging is fascinating. Does this happen in other organs too? While the research primarily examines the human fallopian tube, it would be valuable to extend these observations to other reproductive tissues such as the endometrium, myometrium, ovary, and/or cervix. This expansion could provide insights into the implications of menstruation cessation on immune and stromal/epithelial interactions and what are the potential biological consequences might this have, particularly regarding immune tolerance? Kindly include references to substantiate these assertions.

We agree with the reviewer that it would be valuable if future research could extend these observations to other reproductive tissues, but this is outside the scope of our study. We did not include any statements or speculations in the manuscript regarding reduced cell-to-cell communication between specific cell types and how it could expand to immune cell tolerance or the biological consequences of menopause. We added the following to our discussion to highlight how progesterone is involved in epithelial-stroma crosstalk:

“Progesterone alters stromal cell transition and changes epithelial-stromal crosstalk during embryo attachment and implantation (44). Our data showed increased and strong epithelial-stromal crosstalk/communication during the secretory phase.”

3. Secretory epithelial cells change more significantly than other cell types between pre- and post-menopausal donor samples.

a. What is the rationale behind researchers opting to stimulate primary fallopian tube epithelial (FTEC) cells in vitro with estrogen (E2) and progesterone (P4), rather than utilizing FT epithelial organoids to assess the functionality of secretory epithelial cells (SE cells)? As suggested, the in vitro systems developed from primary cells might not accurately mirror the complex dynamics observed in tissues. Given this limitation, and acknowledging the authors' hard work, one might argue that using epithelial organoids could have been a preferable option, as previously demonstrated by Kessler et al. in Nat Comm. 2015 or Chang et al. in J Biomed Sci. 2020. This approach would not only enable comparisons between proliferative and secretory phases but also between pre-menopausal and postmenopausal samples, a comparison lacking in this section of the reviewed manuscript.

We have not been able to perform long-term culture of fallopian tube organoids as described in the above studies. Organoid viability was very low, especially when post-menopausal fallopian tube samples were used for the organoid culture. As mentioned in our previous rebuttal letter we have only been able to recapitulate the expression of certain genes during hormone treatments in a co-culture system (epithelial cells and fibroblasts) and included these findings for validation purposes only (Manuscript figure 5G). Epithelial organoids, while being an advanced system (3D), usually do not contain stromal cells and would therefore require extensive validation.

4. During the menstrual cycle, secretory epithelial cells change more than other cell types

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We made the requested changes and added relevant references.

1. While it's accurate that the authors have included a section dedicated to RNA velocity analysis, the details outlined in the rebuttal letter contradict those presented in the manuscript. Please, assess this section to ascertain whether Velocity or scVelo was utilized, and include the relevant references accordingly.

We thank the reviewer for noticing this. We used scVelo for RNA velocity analysis. We updated the methods section accordingly and have included relevant references.

2. Given that you've conducted a thorough velocity trajectory analysis utilizing scVelo, it would be greatly appreciated if you could include, at minimum in the rebuttal letter, the resultant outcomes. This would help confirm whether the impact of gene expression features on kinetic patterns and trajectories has provided any further biological insights.

We presented our velocity analysis results on SE1- and SE2- pre and biological insights in the manuscript. Specifically, we utilized velocity analyses to assess our datasets for evidence of phase transition between SE1- and SE2- pre during the menstrual cycle. In UMAP space, we overlaid the velocity vectors to elucidate the direction of cellular transitions. Notably, RNA velocity vectors in pre-menopausal SE cells formed a circle, indicating their involvement in a cyclic biological process (Fig. 5I). This observation aligns with the menstrual cycle pattern, suggesting a regular, repeating transition between SE cell states throughout the cycle. Additionally, we found that the median latent time was significantly shorter during the secretory phase, indicating accelerated export of mature mRNA to the cytoplasm during this phase (Fig. 5J and S4F). This observation suggests higher transcriptional activity in SE cells during the secretory phase, consistent with findings from CellPhone DB analysis (Fig. 5H).

We have opted not to include velocity analysis results from other cell types in this manuscript, as they distract from the narrative of our study.

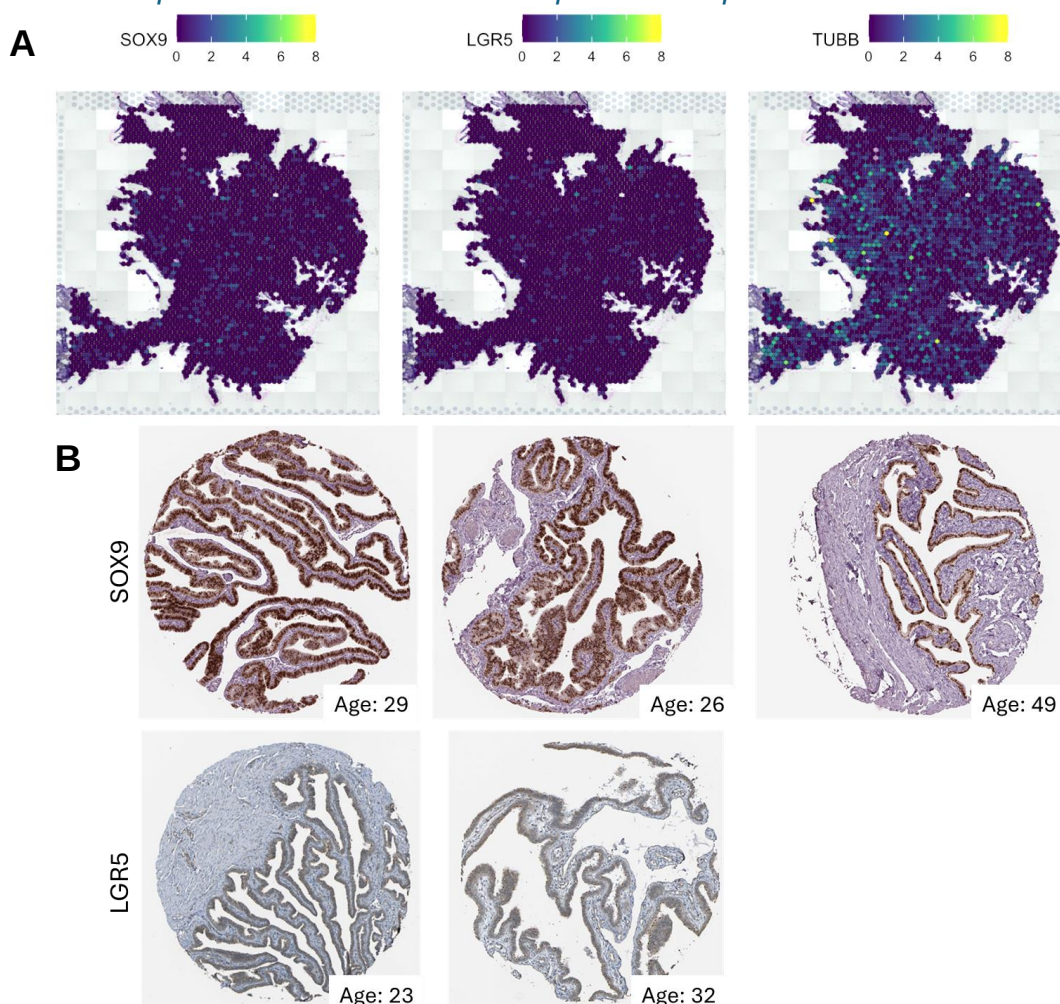
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*We agree with the reviewer that it would be intriguing to investigate the expression of SOX9 and LGR5, but this would require collecting additional samples, and it remains difficult to pre-screen women based on their menstrual cycle phase for fresh-frozen specimen collection. We used our already existing Visium data to look at spatial patterns of SOX9, LGR5, and TUBB expression in the premenopausal fallopian tube fimbria (**Rebuttal Fig. 3A**). Unfortunately, due to the limited resolution of the Visium, no clear spatial patterns of SOX9 and LGR5 expression were observed. Our search of SOX9 and LGR5 expression in the human fallopian tube epithelium revealed that according to the*



Rebuttal Figure 3: A) Spatial transcriptomics profiling of normal pre-menopausal fallopian tube fimbria using 10x genomics Visium. Pre-menopausal fallopian tube fimbria was analyzed for the spatial expression of SOX9, LGR5 and TUBB. B) Immunohistochemistry of SOX9 and LGR5 protein expression in the pre-menopausal human fallopian tube as catalogued by the Human protein atlas. Source: Human Protein Atlas.

*human protein atlas, SOX9 and LGR5 proteins are widely expressed in the fallopian tube epithelium of pre-menopausal women (**Rebuttal Fig. 3B**). Since our SE2-pre cells constitute 84% of SE cells in our cohort we do not anticipate observing any patterns of spatial distribution.*

Reviewer #3 (Remarks to the Author):

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Overall this paper provides strong evidence and is of high importance for the ovarian cancer and Fallopian tube research fields.

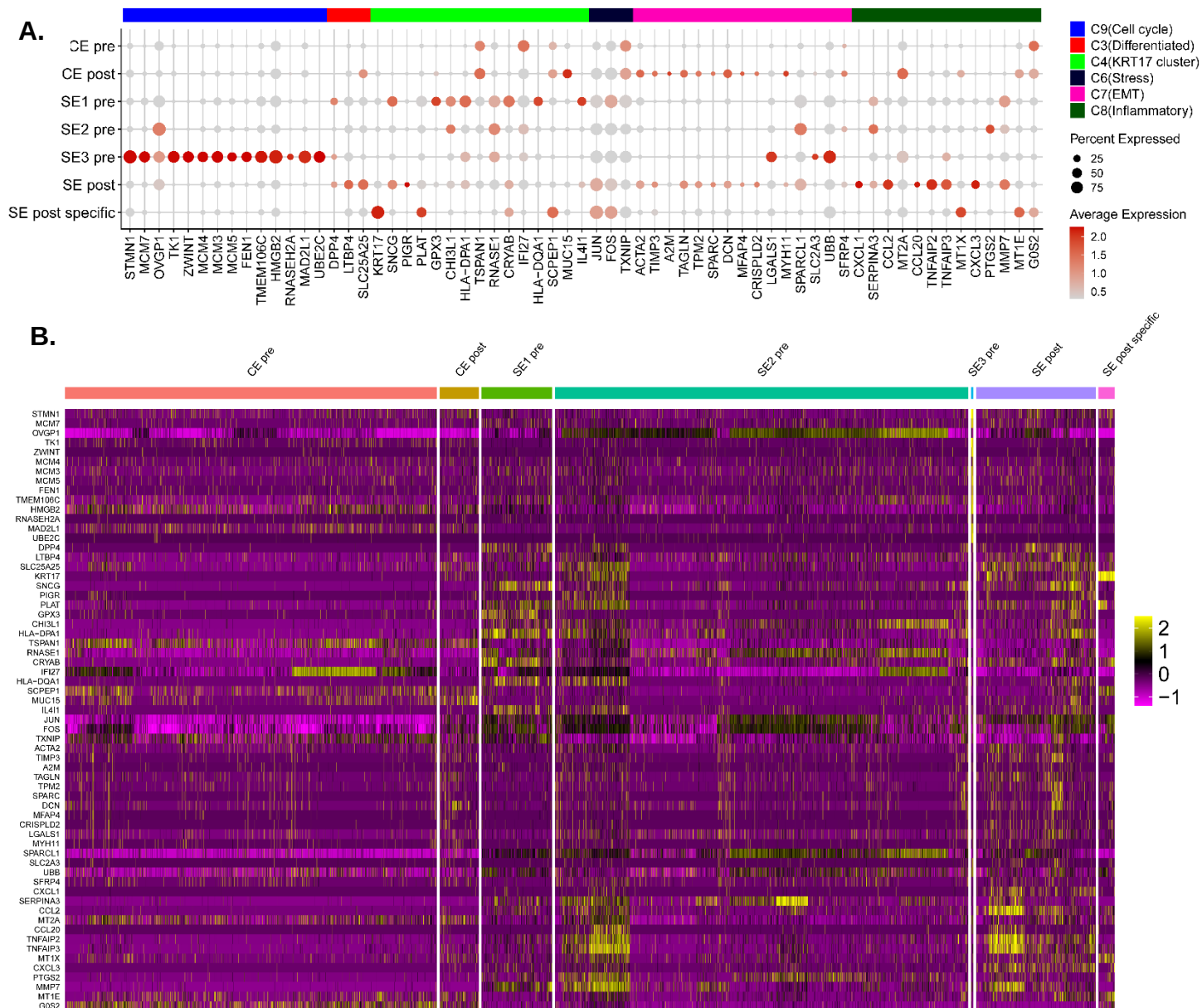
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In spite of the above, I believe that the work is highly valuable and would enrich our understanding of fallopian tube epithelial cell biology in the context of health and disease.

*We thank the reviewer for their feedback and clarification. Unfortunately, we could not recapitulate the cell-clusters previously identified (PMID: 32049047). We assessed the expression of top genes expressed in clusters identified by Hu et al., 2020 in our cell clusters. As shown in **Rebuttal Fig. 4A**, out of the major cell clusters identified by Hu et al., 2020 we only identified cluster 9 (cell cycle cluster), represented by SE3-pre, in our data set. **Rebuttal Fig. 4B** further revealed no clear sub-clusters based on the genes reported by Hu et al., 2020 within our cell types. Since we were not able to identify the recently reported single cell-based cell clusters within our own data set, we choose not to use the new classification when we reported our findings.*



Rebuttal Figure 4: Dot plot (A) and heatmap (B) for top genes upregulated in cell clusters C3 (Differentiated), C4 (KRT17 cluster), C6 (Stress), C7 (EMT), C8 (Inflammatory) and C9 (Cell cycle) based on Figure 2C by Hu et., 2020.

REVIEWERS' COMMENTS

Reviewer #2 (Remarks to the Author):

This interesting report provides crucial insights into the normal physiology and homeostasis of ovarian cancer and the fallopian tube. The authors have thoroughly addressed the concerns raised in the second review implementing the necessary corrections and providing well-reasoned explanations . Overall, this paper supplies robust evidence for this underexplored organ.