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Peer Review File

Time-series single-cell transcriptomic profiling of luteal-phase endometrium uncovers dynamic characteristics and its dysregulation in recurrent implantation failures

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this study, the authors utilized human samples combined with single-cell RNA sequencing to evaluate the cellular landscape of the endometrium during the window of implantation (WOI). The authors established a computational model (StemVAE) for the prediction of temporal changes across the WOI, revealing a two-stage stromal decidualization and gradual transition of epithelial cells during this period. The author then undertook cell-cell communication analysis to further explore the recipients of altered time-dependent signaling in the WOI, and showed strong interaction between stromal cells and immune cells. Based on the generated model, the authors show the recurrent implantation failure (RIF) can be associated with displacement of the temporal WOI-associated changes in stromal and epithelial cells together with an elevated inflammatory microenvironment.

Overall, the data presented herein represent an in-depth characterization of the endometrium that is further strengthened by longitudinal sampling across the WOI, providing a highly comprehensive dataset and computational model that can be leveraged in future investigations. Limitations of the study include a lack of validation for key observations. Moreover, the RIF-related findings warrant further interpretation and dissection. Specific comments are as follows:

Comments

1. Introduction, Lines 54-55: Please be more specific about how compromised crosstalk can cause fertility problems. What is the current evidence?

2. Introduction, Lines 73-74: There are earlier studies that have explored the WOI using single-cell technologies. Please consider citing this work.

3. Results, Lines 119-120: What specific extra steps were taken to minimize variation? Most single-cell dissociation protocols are already designed to minimize any variation.

4. Results, Lines 121-122: It is unclear what the authors mean by “we avoided statistical comparison”? It seems that the authors have sufficient sample sizes for basic two-group statistical comparisons. Please elaborate.

5. Results/Extended Data 3a: In lines 142-143, it is not clear that the spatial data are obtained from a different dataset. Please include this information here.

6. Results, Lines 202-203: I would suggest changing the language here. There are some small shifts/differences between the epithelial and stromal cells only models of StemVAE shown in Ext. 5b.

7. Results, Figure 2b: The LH7 and LH9 peaks seem to show some similarity, is this expected?

8. Results, Lines 209-212: It appears that only the Unciliated epithelial cells have a relatively high correlation, rather than all epithelial. Also, some immune cell subsets such as the NK/T cells have similar correlations with some non-immune subsets, such as Endothelial and Ciliated.

9. Results, Extended Data 6c: Could the authors create a combined plot to allow for direct comparison of the cell clusters between datasets? Based on the language in Lines 228-232.

10. Results, Figure 3a: For the functional enrichment analysis, the authors may want to explore alternative and/or more selective means of showing top processes, as some (like "inflammatory bowel disease") seem out of place at first glance.

11. Results, Lines 283-284: Has SenMayo been validated across reproductive tissues such as the endometrium? Key senescence-related genes may be expected to remain fairly consistent, but validation is needed.

12. Results, Lines 285-289: The endothelial subset seems to show more elevated senescence scores at all time points relative to the epithelial subsets, but this is not mentioned.

13. Results, Lines 289-291: What is the "inflammation score" based on?

14. Results, Figure 5: The authors have plotted the changes in the expression of receptors/ligands on immune cell subsets, but not the concomitant changes in the expression of the corresponding ligand/receptor on non-immune subsets. Are these changes mirrored? Would be interesting to see the side-by-side comparison.

15. Results, Figure 5: Although the authors note that the immune cell subsets are largely absent of notable time-dependent changes, were there any notable interactions (receptor-ligand pairs) that changed during the WOI?

16. Results, Figure 6: Although not apparent from the overall view in Figure 6a, were there any changes in the relative abundance of major clusters or subclusters between controls and RIF?

17. Results, Figure 6b: What cut-off are the authors using for the displacement? For epithelial cells, there also appears to be some modest displacement for RIF-2, -7, and -3 in the opposite direction.

18. Results, Figure 6c: Although not significant, there is a decidualization and senescence score increase in the RIFclass1. Could this reflect a dysfunctional decidualization process in a subset of RIF patients?

19. Results, Figure 6: Did the authors perform any validation of the 9 genes shared among both RIF classes? It would be interesting to see how these results hold up in fresh samples and/or other datasets.

20. Results, Line 431: The cited reference is based on peripheral monocytes in the context of COVID-19, sepsis, or HIV infection. Do the authors consider that their myeloid population is primarily newly infiltrated monocytes from the circulation? Perhaps a more closely related reference dataset could be utilized here.

21. Results, Figure 6f: Why would Tregs have such high expression of IFNG?

22. Results, Figure 6e & g: The UMAPs here should include a reference map to aid readers in remembering cluster identities.

23. Results, Figure 6: Similar to the above comments, did the authors validate the increased expression of IFNg by NK and T cells? Have other studies shown excessive IFNg production by NK and T cells associated with RIF?

24. Discussion: The interpretation of the findings derived from applying the StemVAE model to the RIF cases is not clear. What is the takeaway from observing the displacement of epithelial and stromal signatures in the RIF cases? The authors seem to primarily focus on which genes show altered expression, but the timing of the changes does not seem to be highly touched on here despite being a major focus of the rest of the manuscript. The authors would do well to expand on future directions and how they or other investigators can apply the model/data generated herein. In addition, please include a limitations section.

25. Figures: In general, additional labelling/titles for each plot type across the figures would help the reader identify what is being shown in each panel.

26. Figure 1: Figure 1b could be better organized, having the major populations in the middle and the subpopulations around is not easy to follow at first glance. Additional labels in the figure would also help.

Reviewer #2 (Remarks to the Author):

The authors conduct an extensive analysis of individual cells to examine the dynamics of the endometrium throughout the window of implantation (WOI). This comprehensive atlas, comprising 220,000 cells, offers valuable insights for investigating recurrent implantation failure (RIF) and other endometrial pathologies.

However, it's crucial to acknowledge that many assertions in this study rely solely on computational analysis and inference drawn from the single-cell dataset. Further validation through orthogonal methods is essential to support these claims.

- Sample preparation for single cell data: tissue dissociation and stress response. Sample preparation and cell isolation protocols are essential for high quality data and faithful representation of all components of the tissue, especially when authors evaluate cell compositions (line 106-119) and time course analysis for specific cell types. The authors used a 1h long, 1 step

digestion protocol. Other studies have shown the value of gentle protocols to minimize stress response and provide a faithful representation of all cell types/states, closed to initial tissue composition. Did the authors compared the single cells obtained post dissociation to the tissue pre-dissociation? Is there any missing cell type? Authors mentioned genuine tissue variation – can you illustrate this with H&E representation of the sampled tissues?

Extensive and stringent digestion can impact drastically the transcriptomic signature too. In this case where authors chose a long digestion, it is particularly concerning since some of the key markers for cells populations are related to stress response-induced genes such as JUN/FOS like in stromal cells (Str3) or immune cells (NK, T cells in the StemVAE analysis). As well, DEG lists show the presence of mitochondrial genes and unannotated sequences or LncRNA within the top DEG. Can the author analyze the dissociation-induced stress response across different cell types/subtypes?

- The analysis of the endometrium during the WOI is very interesting. However little information is provided around LH peak and sampling of the biopsies. Did the authors check LH levels at LH+1, +3,... to verify that all samples biopsies were collected at similar times related to the LH peak/individual? Were all LH peaks similar across patients?

- Cell type annotations EMT/MET: I find concerning that the authors claim the presence of EMT/MET cells without any further validation (IHC/IF at least should be used to confirm the existence of these cells in the tissue). How did the author discard the presence of doublets and potential mislabeling of these cells? Particularly, can the authors plot the EMT/MET cells back onto the main clustering? In the provided UMAP, a stromal (pink colored) subpopulation seem to cluster very closely from epithelial cells- is this the MET cluster?

- The integration of spatial transcriptomics (Visium), albeit at a resolution different from single-cell analysis, was attempted using data from a single experiment and endometrial biopsy from a published manuscript, likely not corresponding to the time points analyzed in this study.

I would suggest to validate cell populations, markers and CCC with a spatial transcriptomics/proteomic assay, single cell resolution if possible, using comparable samples and justifiable n.

- CCC analysis: are the L/R interactive pairs validated across all patients?

- Senescence: authors combined all cell subpopulations within a specific cell type to evaluate the putative senescence signature during the WOI - using SenMAyo, a senescence signature derived from human/mouse adipose tissue and bone marrow (not validated in teh endometrium). Does this apply to all stromal cell clusters? Surely there are key differences between stomal cell

subpopulations, but do they all play a similar role in WOI and display similar senescence markers? Orthogonal validation would be good to confirm any of these claims

- I would suggest to refrain from statements such as “Altogether, the continuous elevation of the senescence score from LH+7 supported that the stromal senescence tunes the endometrial inflammatory microenvironment.” At best, the analysis shows association, and without any orthogonal validation or functional validation/mechanistic study, this looks like an overstatement. Same for sentence line 298- authors have shown association, but not correlation.

- LGR5+ cells: authors suggest LGR5 is expressed in luminal/progenitor epithelial cells, but gene expression data shows no expression of LGR5 in the luminal compartment (Ext fig 2g). Additionally, the IF image (fig 1) suggest that LGR5 is also present in stromal cells, in addition to epithelial cells. Are those luminal or glandular cells? can you provide further details about RNA/protein detection of LGR5 within endometrial cells?

- Extended Fig 3d: how stable is this tree and subjected to variable cell numbers for each category? What do the top numbers correspond to?

- Line 341- CDH1 is defined as a cell-cell adhesion molecule specific to epithelial cells and its loss has been shown to induced epithelial cell disorganization and as a consequence is linked to infertility in mouse studies. Can authors provide human-based literature to show that it is a "known" regulator of implantation?

- Can the authors confirm the expression of HLA-DOB in epithelial cells through orthogonal validation?

- The computational analysis suggest or predicts but without any validation (functional/orthogonal) this is not a demonstration (line 413). This type of statement comes through often in this manuscript and I would suggest to carefully use such statement when driven by computational inference only.

- While author conduct a thoughtful computational analysis of the immune/myeloid cells within RIF samples based on the obtained single cell data, they do not provide any functional

validation/orthogonal validation to sustain the claims of correlation or hyperinflammation, nor the implication in impaired receptivity for these particular cell types.

Reviewer #3 (Remarks to the Author):

This manuscript reports on the uterine endometrial transcriptome at single cell resolution across the secretory phase of the menstrual cycle in fertile volunteers (controls) and in the window of implantation (WOI) in cases with a history of recurrent implantation failure (RIF). The data from controls add to recent datasets with novelties being that samples were obtained timed to the LH surge before, during, and after the WOI, and application of an algorithm, StemVAE, developed by the authors to model time-dependent endometrial single cell data across the secretory phase. Several key findings in controls without RIF include interpretation of the data that reflects a gradual transition of epithelial cell gene dynamics across the WOI, in contrast to an abrupt transition of stromal cells at LH+7 (the opening of the WOI) and minimal changes in other cell types; identifying luminal-glandular characteristics of luminal epithelial cells; identifying a set of epithelial receptivity genes, identifying epithelial and stromal cell cross-communication with immune cells, limited inflammatory features in the WOI and more robust inflammation beyond the WOI (consistent with the tissue preparing for menstrual shedding), and that senescent stromal cells may control inflammation. The RIF WOI samples were classified as “displaced” or “dysregulated” based on differences mainly in epithelial cluster profiles and especially immune cells that were characterized by a hyper-inflammatory environment in the “dysregulated” phenotype, compared to controls. Overall, the manuscript provides a valuable dataset of endometrial cell dynamics across the secretory phase of the menstrual cycle. However, several items warrant further clarification to assure rigor of the results, interpretation of some of the data, and to support some of the conclusions drawn.

Specific Comments to be addressed:

1. While cases and controls were fairly well-matched by BMI and age, it is important to include in Supplemental Data 1 the gravidity and parity of the donors (i.e., how many pregnancies, miscarriages, live births each had). Prior pregnancy may impact endometrium in various ways, and so this needs to be considered and evaluated in terms of the marked heterogeneity of the data among controls and in differences between RIF versus controls (see below).

2. For the RIF patients, were the endometrial biopsies evaluated for endometritis, a common cause of RIF? This would be to document if CD138+ plasma cells were found in the endometrium, and if so were patients treated? Was this also evaluated in controls? This is essential to perform in the RIF samples, as the data demonstrated intense endometrial inflammation in the RIF WOI. Where might

this come from, as some disorders predisposing to endometrial inflammation (e.g., infection, endometriosis and other) were apparently among exclusion criteria for donors?

3. How do the authors know donors and RIF volunteers did not have endometriosis, adenomyosis, endosalpingiosis, hypdrosalpinx, or other conditions that are associated with an inflammatory milieu in the endometrium – especially in RIF cases and also in controls, which were mainly "public". Did they have imaging and/or surgery to assess these conditions that affect the endometrium?

4. Figure 1b: transcriptomic dimensional reduction revealed epithelial luminal, secretory, glandular, and proliferative subpopulations. To what does the proliferating population refer – proliferating, cycling cells? And the secretory cells - are those luminal, glandular, and all unciliated? Please clarify.

5. Figure 1c: it is surprising that epithelial cell markers do not include FOXO1, as it is a major contributor to epithelial integrity and is a core transcription factor of epithelium involved in endometrial receptivity. Please explain.

6. Figure 1e: the validation is done in proliferative endometrium (before the LH surge). It should be in secretory endometrium (when the samples were obtained for scRNA seq).

7. Extended data Fig 2. The extensive inter-individual abundance of various cell types across all samples is attributed by the authors to intrinsic differences in the various tissues. Is this reproducible from cycle to cycle within the same donor? Also, the vast heterogeneity could also be due to prior history of pregnancy or different hormonal milieu at a given time point post LH surge. Having serum estradiol and progesterone levels are key to determine if hormone levels could contribute to this heterogeneity in this highly steroid hormone-responsive tissue and its constituent cells, in addition to histology of the tissues at given time point. Also, different regions of the endometrium being sampled for analysis could account for this heterogeneity – e.g., is an epithelial cell adjacent to an immune cell the same as if it were near a fibroblast?

8. Figure 2b needs more explanation in the legend and some labeling in the figure per se.

9. Figure 2d: M&M says n=3 from each group at LH+3, LH+5, LH+9, LH+11 and n=10 from LH+7. The group ratios (different colored bars) don't reflect this at the bottom of the panel (and in other figures, too). Please explain.

10. How did Stem-VAE account for differences in cell abundances for all the samples?

11. Stem-VAE revealed dynamic changes of the epithelial cells and stromal cells that highly correlated with time across the WOI, but the immune cell features appeared to be time-invariant. This is in stark contrast to histologic data of immune populations changing across the cycle, and especially beyond the WOI when the tissue prepares for shedding, which the authors also found. This seems inconsistent. Please explain.

12. While epithelial glandular, luminal, secretory, and EMT subtypes displayed differential time-associated gene expressions, the ciliated, EPCAM1+SOX9+, LGR5+, and proliferative subpopulations did not. What are the "proliferative" subpopulations?

13. In Figure 3, stromal fibroblast profiles in LH+3 and LH+5 look very similar, consistent with bulk transcriptomic data by others. Important to cite these supportive bulk RNA seq or microarray studies done by others.

14. In Figures 3 and 4, as the authors mention, the data differ from those of the Wang et al endometrial scRNA seq study across the menstrual cycle who found that the epithelial profiles abruptly open the WOI; whereas, the stromal fibroblast transition is not abrupt. Herein, the Figure 4 dendrogram shows that genes for 2 sub-branches of the distal right branch on the x axis are highly expressed in LH+3 and LH+5 and then abruptly diminish thereafter, opening the WOI, although the genes are different than in Wang et al. In Figure 3 the effect is even more dramatic. The authors suggest their sampling timed to the LH surge, not done in the Wang study, may account for these discrepancies. However, there may be other reasons including different patient characteristics (e.g., prior pregnancies or not, different hormonal milieu, different endometrial histology – again another reason to have the latter 2 items in the clinical metadata and do sub-analyses of the scRNA seq data herein. Also, this should be considered in the Discussion section.

15. The WOI is generally considered to span LH+7 to LH+10 for embryo attachment and beginning breaching the epithelium into the stromal compartment. How do the authors reconcile that the epithelial dynamics in Fig 4 show that LH+9 variably resembles LH+7 and the 2 sampling points reveal quite different profiles in the stromal population in Fig 3 with these processes? What is the model in terms of known histology and WOI duration which is an approximation but still spans ~ 4 days?

16. How do the epithelial receptivity genes correlate with those in bulk datasets (e.g., Altmae 2017), and the clinical ERA bulk transcriptome test or the immunohistochemical clinical BCL6 test for inflammation or other epithelial markers of receptivity (e.g., beta 3 integrin)?

17. scRNA seq data from the 10 RIF samples resulted in classification of 2 groups: Class 1 with “displacement” of the WOI and Class 2 with “dysregulated” WOI. The main driver was epithelial receptivity scores, and Class 2 samples displayed a hyper-inflammatory microenvironment involving myeloid cells with high upregulation of IFN-□ in NK and T cells and IFN-□R1 in luminal epithelial cells. This is a remarkable set of observations, consistent with bulk data studies (should also cite refs from Macklon’s group, including PMC4716345).

18. With regard to the RIF Class 1 “displacement group” – which way is the window displaced – to a pre-receptive or post-receptive profile?

19. With regard to “dysregulated” Class 2, the inflammatory profile warrants further investigation about the patients – see Comments 2 and 3 above about infection and inflammatory disorders.

20. Commonly associated with endometrial inflammation is abnormal progesterone signaling in stromal fibroblasts and in epithelium. Did the authors detect abnormal downstream events from PR in Class 2 RIF samples?

21. Also, notably in Figure 6a, it appears that there are more B cells in the RIF group(s) – is that correct? If so, the possibility of infection arises. Also, any differences in the B cells between RIF Class I and Class II samples?

22. Other items:

a. When referring to stromal cells, the correct nomenclature is ““stromal fibroblasts”. Please correct throughout the manuscript.

b. Please put the # of RIF samples in the Materials and Methods (only the number of controls is listed there).

c. Figure 1b, central portion – for clarity, arrows or lines connecting the subtypes in the periphery to the central portion would be helpful.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this study, the authors utilized human samples combined with single-cell RNA sequencing to evaluate the cellular landscape of the endometrium during the window of implantation (WOI). The authors established a computational model (StemVAE) for the prediction of temporal changes across the WOI, revealing a two-stage stromal decidualization and gradual transition of epithelial cells during this period. The author then undertook cell-cell communication analysis to further explore the recipients of altered time-dependent signaling in the WOI, and showed strong interaction between stromal cells and immune cells. Based on the generated model, the authors show the recurrent implantation failure (RIF) can be associated with displacement of the temporal WOI-associated changes in stromal and epithelial cells together with an elevated inflammatory microenvironment.

Overall, the data presented herein represent an in-depth characterization of the endometrium that is further strengthened by longitudinal sampling across the WOI, providing a highly comprehensive dataset and computational model that can be leveraged in future investigations. Limitations of the study include a lack of validation for key observations. Moreover, the RIF-related findings warrant further interpretation and dissection. Specific comments are as follows:

Comments

R1.1. Introduction, Lines 54-55: Please be more specific about how compromised crosstalk can cause fertility problems. What is the current evidence?

Response: The importance of blastocyst-endometrium crosstalk on implantation is well documented. Intrauterine infusion of spent culture medium of implanted human embryos enhances expression of metabolic and implantation related genes, whereas those from poor quality embryos induces stress response of the mouse endometrium [1]. The human endometrial stromal cells are biosensors of the embryo quality [2] and respond to embryo-derived hsa-miR-320a by modifying their migratory capacity towards high quality blastocysts [3] and to a serine protease from poor quality embryos by reducing the production of implantation related factors [4]. Secretion of the poor-quality embryos also abolished the killing of senescent endometrial stromal cells by uterine natural killer cells [5]. The biosensing function of the decidualized endometrial stromal cells has been proposed as one of the implantation checkpoints, and its impairment may account for the maternal age-independent stepwise increase in miscarriage rates after each pregnancy loss [6]. On the other hand, embryo attachment onto endometrial epithelial cells promotes differentiation of syncytiotrophoblast from [7] and expression of adhesion molecules on [8] the trophectoderm of human blastocysts. Endometrium-derived exosomal microRNA let-7 induce diapause of mouse blastocysts and

inhibit trophoblast differentiation in a human embryo surrogate model [9].This information was added into the revised manuscript as suggested (line 54-67).

R1.2. Introduction, Lines 73-74: There are earlier studies that have explored the WOI using single-cell technologies. Please consider citing this work.

Response: In addition to the following two publications about WOI endometrium cited in our original manuscript, PubMed search reveals one more report, which used single-cell transcriptomic sequencing to analyze endometrium from peri-implantation window (*Lucas et al, 2020*)[10]. This was properly cited in the revised manuscript (line 86-87). If we omitted other related studies, please kindly let us know.

a. Wang W, Vilella F, Alama P, Moreno I, Mignardi M, Isakova A, Pan W, Simon C, Quake SR. Single-cell transcriptomic atlas of the human endometrium during the menstrual cycle. Nat Med. 2020 Oct;26(10):1644-1653. doi: 10.1038/s41591-020-1040-z.
b. Lai ZZ, Wang Y, Zhou WJ, Liang Z, Shi JW, Yang HL, Xie F, Chen WD, Zhu R, Zhang C, Mei J, Zhao JY, Ye JF, Zhang T, Li MQ. Single-cell transcriptome profiling of the human endometrium of patients with recurrent implantation failure. Theranostics. 2022 Sep 6;12(15):6527-6547. doi: 10.7150/thno.74053.

R1.3. Results, Lines 119-120: What specific extra steps were taken to minimize variation? Most single-cell dissociation protocols are already designed to minimize any variation.

Response: Yes, we agree that most single-cell dissociation protocols are optimized. In this study, we took special precautions to minimize variation in sample collection. First, the same doctor (YF.C.) collected all the endometrium biopsies, and the same technician (J.W.) processed the biopsies according to our standard operation procedures. Second, endometrial dating was done by determination of serum luteinizing hormone level. We added this information into the Methods (line 701-707, line 737-738).

R1.4. Results, Lines 121-122: It is unclear what the authors mean by "we avoided statistical comparison"? It seems that the authors have sufficient sample sizes for basic two-group statistical comparisons. Please elaborate.

Response: In experimental biological studies, a sample size of 3-5 is often sufficient because most of the experimental variables including animals and cells are assumed to be well controlled. This assumption is unsuitable in clinical studies due to large inter-individual variations. The cellular composition of endometrial biopsies is known to exhibit substantial variations [11,12] and statistical comparison of the small number of samples (3 for LH3/5/9/11, 6 for LH7, 10 for RIF) in this study may not truly reflect the differences between groups. Therefore, we did not perform analyses comparing cellular composition of

different groups of biopsies. We've rephrased the words to make clearer elaboration in the revised manuscript (line 138-140).

R1.5. Results/Extended Data 3a: In lines 142-143, it is not clear that the spatial data are obtained from a different dataset. Please include this information here.

Response: This was clarified in the revised manuscript by stating "by utilizing a public spatial transcriptomic dataset of secretory human endometrium" and proper citation of the related publication (line 155-156).

R1.6. Results, Lines 202-203: I would suggest changing the language here. There are some small shifts/differences between the epithelial and stromal cells only models of StemVAE shown in Ext. 5b.

Response: To elaborate accurately as suggested, we changed the description to "The predicted time distribution domain mostly remained within the same interval when StemVAE was built solely on the epithelial cells or the stromal cells" in the revised manuscript (line 232).

R1.7. Results, Figure 2b: The LH7 and LH9 peaks seem to show some similarity, is this expected?

Response: The WOI is generally considered to span 2~4 days from LH+7 [13] and covers samples for LH7 and LH9. Therefore, it is reasonable to observe some similarity between the LH7 and LH9 peaks in Figure 2b. In addition, the dynamic gene expression patterns of stromal cells and epithelial cells showed similar expression for subset of the cells at the two time-points especially the stromal cells (Figure 2d, Figure 3&4), consistent with the time distribution similarity for the LH7 and LH9 peaks in Figure 2b.

R1.8. Results, Lines 209-212: It appears that only the Unciliated epithelial cells have a relatively high correlation, rather than all epithelial. Also, some immune cell subsets such as the NK/T cells have similar correlations with some non-immune subsets, such as Endothelial and Ciliated.

Response: Yes. Only unciliated epithelial cells and stromal cells showed high correlation with Spearman's rank correlation coefficient around 0.8. while the remaining cell subtypes showed moderate or low correlation. We modified the result description by changing the 'epithelial cell' to 'unciliated epithelial cells' and including the remaining subsets as suggested. The final sentence is modified as below in the revised manuscript (line 240-244).

"The results supported our speculation that the dynamic changes of the unciliated epithelial cells and the stromal cells were highly correlated with the time while that of the immune subsets as well as ciliated epithelial cells and endothelial cells showed moderate (around 0.5) or low (less than 0.5) time-association

irrespective of the gene sets (HVGs or time-associated genes identified by variancePartition) that StemVAE was built on".

R1.9. Results, Extended Data 6c: Could the authors create a combined plot to allow for direct comparison of the cell clusters between datasets? Based on the language in Lines 228-232.

Response: Extended Fig 6c was also based on our dataset rather than another dataset but was analyzed with a different method named psupertime which uses cross-entropy loss. The information delivered here was to confirm that the non-immune subsets from different time points had highly overlapped predicted time while the stromal and unciliated epithelial cells had more separated predicted time. This was supportive of what was displayed in Fig2b-d and Extended Fig 6a,b from our method StemVAE which uses mean-squared loss.

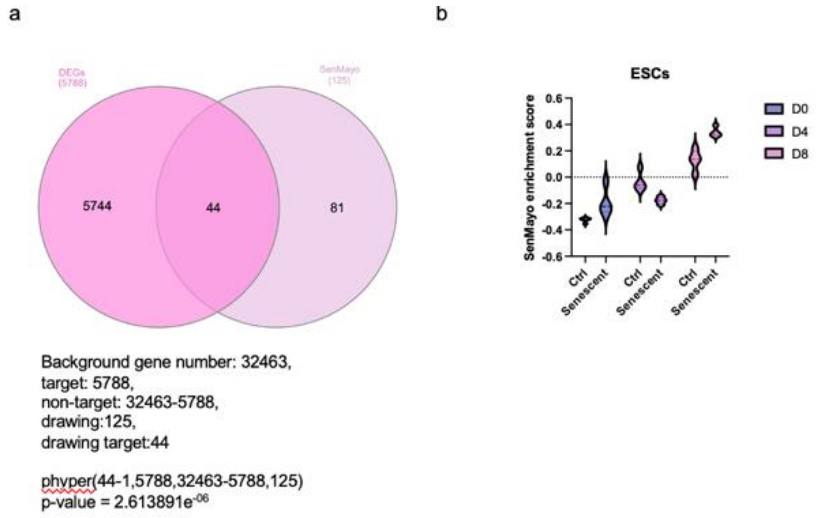
R1.10. Results, Figure 3a: For the functional enrichment analysis, the authors may want to explore alternative and/or more selective means of showing top processes, as some (like "inflammatory bowel disease") seem out of place at first glance.

Response: Thank you for pointing out this issue. We used Enrichr which is widely used to perform comprehensive functional enrichment analysis using Reactome/KEGG/MSigDB databases. All significant enriched terms from the three databases with an adjusted p value < 0.05 are listed in Supplementary Table 3. Figure 3a shows selected processes that we considered as most related. The selected 'inflammatory bowel disease' was intended to show the inflammatory response rather than the bowel disease. To avoid misunderstanding, we replaced this term by displaying the most significantly enriched KEGG pathway 'Longevity regulating pathway'.

R1.11. Results, Lines 283-284: Has SenMayo been validated across reproductive tissues such as the endometrium? Key senescence-related genes may be expected to remain fairly consistent, but validation is needed.

Response: To validate the SenMayo gene set in reproductive tissues, we performed two additional analyses. First, we retrieved differentially expressed genes (DEGs) identified from a published study that compared young and aged human endometrium [14]. Hypergeometric test showed that the SenMayo gene set were significantly enriched in the DEGs (Panel a of the figure below). Second, we used a public dataset of human endometrial stromal cells that were induced to senescence by H_2O_2 treatment [15]. GSVA analysis of the SenMayo gene set was applied to the dataset and found that the senescent stromal cells had elevated enrichment score when compared to the untreated one (Panel b of the figure below). These results collectively showed that the SenMayo gene set could be used for the senescence analysis in the human

endometrium. These validation results were displayed in Supplementary Figure 5 and the validation process was described in the Methods section of the revised manuscript (line 336-327, line 793-798).



R1.12. Results, Lines 285-289: The endothelial subset seems to show more elevated senescence scores at all time points relative to the epithelial subsets, but this is not mentioned.

Response: The enhanced senescence score was mentioned together with the stromal cells which was stated as "The results showed enhanced senescence score (above the 0 line) in the stromal cells and the endothelial cells". To make comparison to the epithelial subsets, we have modified the sentence by adding "when compared to epithelial subsets" in the revised manuscript (line 327-328).

R1.13. Results, Lines 289-291: What is the "inflammation score" based on?

Response: Sorry to miss the related information. The inflammation score was calculated in the same way as the senescence score by using a published inflammatory gene list. We have properly mentioned the calculation for 'inflammation score' in the Results section (line 334-335) and modified the Methods section (line 793-798) in the revised manuscript.

R1.14. Results, Figure 5: The authors have plotted the changes in the expression of receptors/ligands on immune cell subsets, but not the concomitant changes in the expression of the corresponding ligand/receptor on non-immune subsets. Are these changes mirrored? Would be interesting to see the side-by-side comparison.

Response: In Figure 5, Figure 5a and Figure 5d respectively depicted the dynamic changes of the paired ligands/receptors in stromal and epithelial cells. To facilitate comparison, we re-plotted the subfigures showing side-by-side paired ligands/receptors expression using the violin plots (Figure 5c,f).

R1.15. Results, Figure 5: Although the authors note that the immune cell subsets are largely absent of notable time-dependent changes, were there any notable interactions (receptor-ligand pairs) that changed during the WOI?

Response: In our analysis, we firstly restricted significantly changed ligands/receptors in the non-immune cells. Then, the significant interacted ligand/receptor pairs were selected as shown in Figure 5b,e. Therefore, the finally identified ligand/receptor pairs (as shown in updated Figure 5c,f) were putative changing interactions during the WOI.

R1.16. Results, Figure 6: Although not apparent from the overall view in Figure 6a, were there any changes in the relative abundance of major clusters or subclusters between controls and RIF?

Response: As stated in the first part in the Results section, the cell abundance varied a lot across samples (Extended Fig. 2) which might cause insignificant comparisons with the current small sample size. Instead of comparing the cell percentages across individuals, fold enrichment analysis (taking the total cell type distribution within each group as the reference) was conducted and revealed an enrichment of myeloid cells/endothelial cells and a depletion of epithelial cells when compared to the LH7 controls (Supplementary Figure 10b). The myeloid enrichment was mentioned in the Results (line 504-506, highlighted).

R1.17. Results, Figure 6b: What cut-off are the authors using for the displacement? For epithelial cells, there also appears to be some modest displacement for RIF-2, -7, and -3 in the opposite direction.

Response: Thank you very much for this critical comment. We defined the displacement group mostly based on the percentage clustering result shown in the heatmap (Figure 6b) instead of setting a hard cutoff. As shown in the figure, RIF-2, -7, and -3 clustered together in the tree and showed modest epithelial cell displacement. But on a higher order, they clustered with RIF-1, -6, -5, -9, while RIF-4, -8, -10 remained as a discrete cluster showing their strong displacement. Meanwhile, RIF-8, -10 also demonstrated strong stromal cell displacement. Taken together, we categorized RIF-4, -8, -10 as the displacement group. In the future, with more clinical samples, we will upgrade our method and set standards tailored for translational use. This has been discussed in the revised manuscript (line 629-632).

R1.18. Results, Figure 6c: Although not significant, there is a decidualization and senescence score increase in the RIFclass1. Could this reflect a dysfunctional decidualization process in a subset of RIF patients?

Response: Yes, this is possible and consistent with the result that the RIFclass1 was displaced to a later time interval during which the decidual and senescent related gene expression (e.g. FOXO1, DKK1) should be higher according to the dynamic expression change pattern revealed in Figure 3. We added the description in the Results (line 467-469).

R1.19. Results, Figure 6: Did the authors perform any validation of the shared among both RIF classes? It would be interesting to see how these results hold up in fresh samples and/or other datasets.

Response: We only had spare cell mixtures after single-cell encapsulation for a small subset of the samples which was not suitable for validation of the 9 genes associated with epithelial-dysregulation for both classes. As suggested by this comment, we alternatively performed in-silico validation using two additional public single-cell datasets (GSE183837, GSE223672) of RIF [16,17]. Integration analysis of the two datasets identified DEGs between the RIF and the control endometria. The results showed that 7 of the 9 genes, were significantly dysregulated in the RIF endometrial epithelial cells. Consistently, the expression of DEFB1 and S100P exhibited a decreasing trend though the change did not yet reach statistical significance. This validation analysis was described and added as Supplementary Figure 9 in the revised manuscript (line 491-492).

R1.20. Results, Line 431: The cited reference is based on peripheral monocytes in the context of COVID-19, sepsis, or HIV infection. Do the authors consider that their myeloid population is primarily newly infiltrated monocytes from the circulation? Perhaps a more closely related reference dataset could be utilized here.

Response: We would like to clarify that although the cytokine list was retrieved from the publication which studied peripheral monocytes, only the gene set termed "HALLMARK_INFLAMMATORY_RESPONSE" from MSigDB was used in our study. This gene set represents universal inflammation and not just for peripheral immune cells. To avoid confusion, we described the source of this gene set instead of citing the study about COVID-19, sepsis, or HIV infection in the Results and Methods sections in the revised manuscript (line 334-335, line 793-798).

R1.21. Results, Figure 6f: Why would Tregs have such high expression of IFNG?

Response: Conventionally, Tregs cells play important role in down-regulating immune responses through their maintenance of self-tolerance and modification of many immune cells function [18-20], including inhibition of IFN-γ synthesis of T cells [21]. IFNG-producing Tregs cells [22,23] have been reported and the Tregs cells express IFNG when they were in a Nrp1-deficient state or induced in the periphery from the $CD4^{+}Foxp3^{-}$ conventional T cells [24,25]. The Tregs cells also acquired expression of transcription factors

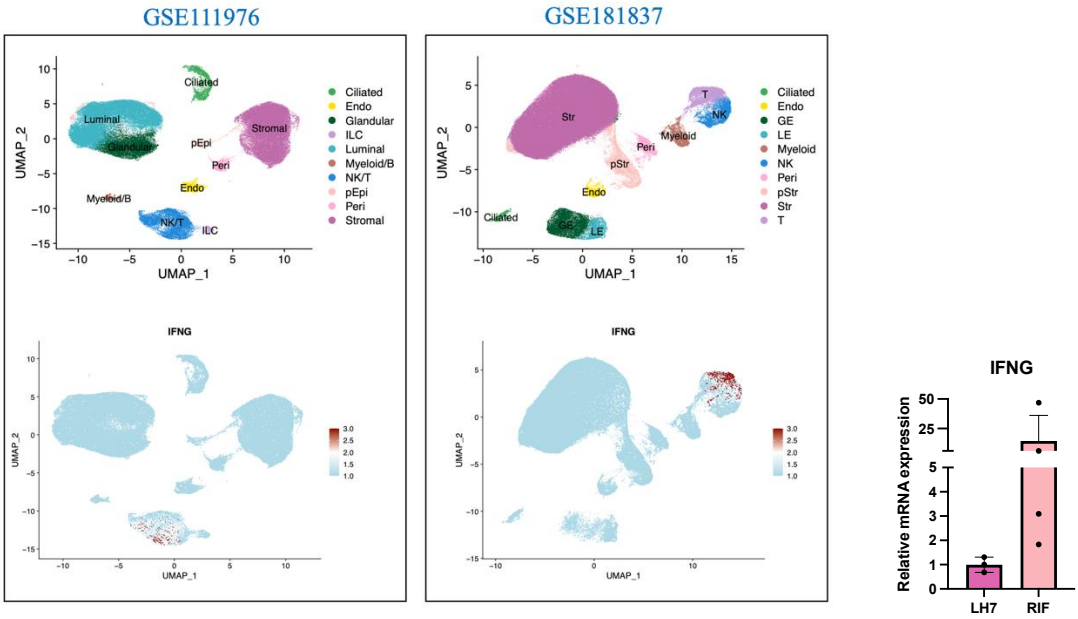
and cytokines, such as T-bet and IRF4, modulating their Th1-homing and Th2-control capacity at the site of inflammation [26,27]. During infection, the Foxp3(+) Treg cells also express IFN-γ [26,28]. Although the Tregs cells express IFN-γ, their immunosuppressive function is not changed significantly in *in vitro* study [29]. Therefore, the high expression of IFNG in the Tregs cells of RIF in our study might be a result of inflammatory response.

R1.22. Results, Figure 6e & g: The UMAPs here should include a reference map to aid readers in remembering cluster identities.

Response: As suggested, the UMAP plot with cell identities of Figure 6g has been added. While the UMAP for Figure 6e can be found in Figure 6a, we chose to mention Figure 6a together with Figure 6e when describing the results instead of including the duplicated UMAP here.

R1.23. Results, Figure 6: Similar to the above comments, did the authors validate the increased expression of IFNg by NK and T cells? Have other studies shown excessive IFNg production by NK and T cells associated with RIF?

Response: In this study, the expression of IFNG was high in NK/T cells, but minimal in epithelial and stromal cells (Figure 6). We additionally analyzed two external single-cell datasets of human endometrium (GSE111976, GSE183837) [12,16]. The results consistently showed the exclusive expression of IFNG in the NK/T cells (Figure below). As IFNG is highly expressed in NK/T cells but not in other cell types, we therefore used qPCR in the total RNA of our spare samples after single-cell encapsulation and found high IFNG level in the RIF group when compared with that of the LH7 (figure below). The result has been added to Supplementary Figure 10 in the revised manuscript. The samples collected in this study were limited and mostly were used for scRNA-seq. On the other hand, recruitment of new samples within a short duration is not feasible. Therefore, future long-term functional and mechanistic study with new samples and models are warranted. Increased expression of IFNG in endometrium [30] and peripheral blood [31] of women with RIF have been reported. However, no studies reported the association of high IFNG production in the NK and the T cells with RIF, though there were studies showing the expression of IFNG in NK/T cells in other systems [32,33]. These were discussed in the revised manuscript (line 663-664).



R1.24. Discussion: The interpretation of the findings derived from applying the StemVAE model to the RIF cases is not clear. What is the takeaway from observing the displacement of epithelial and stromal signatures in the RIF cases? The authors seem to primarily focus on which genes show altered expression, but the timing of the changes does not seem to be highly touched on here despite being a major focus of the rest of the manuscript. The authors would do well to expand on future directions and how they or other investigators can apply the model/data generated herein. In addition, please include a limitations section.

Response: Thank for very much for the very constructive advice. The discussion about the displacement should have been primarily emphasized. As suggested, we expanded the discussion by including thorough discussion about the application of StemVAE model in the identification of displacement, current limitations, and future directions for the improvement (line 598-615).

R1.25. Figures: In general, additional labelling/titles for each plot type across the figures would help the reader identify what is being shown in each panel.

Response: To improve the readability, we have modified Figure 1b and Figure 5, and added titles to several subfigures. Meanwhile, the figure legends were also modified.

R1.26. Figure 1: Figure 1b could be better organized, having the major populations in the middle and the subpopulations around is not easy to follow at first glance. Additional labels in the figure would also help.

Response: Figure 1b is reorganized with additional labels in the revised manuscript.

Reviewer #2 (Remarks to the Author):

The authors conduct an extensive analysis of individual cells to examine the dynamics of the endometrium throughout the window of implantation (WOI). This comprehensive atlas, comprising 220,000 cells, offers valuable insights for investigating recurrent implantation failure (RIF) and other endometrial pathologies.

However, it's crucial to acknowledge that many assertions in this study rely solely on computational analysis and inference drawn from the single-cell dataset. Further validation through orthogonal methods is essential to support these claims.

R2.1- Sample preparation for single cell data: tissue dissociation and stress response. Sample preparation and cell isolation protocols are essential for high quality data and faithful representation of all components of the tissue, especially when authors evaluate cell compositions (line 106-119) and time course analysis for specific cell types. The authors used a 1h long, 1 step digestion protocol. Other studies have shown the value of gentle protocols to minimize stress response and provide a faithful representation of all cell types/states, closed to initial tissue composition. Did the authors compared the single cells obtained post dissociation to the tissue pre-dissociation? Is there any missing cell type? Authors mentioned genuine tissue variation – can you illustrate this with H&E representation of the sampled tissues?

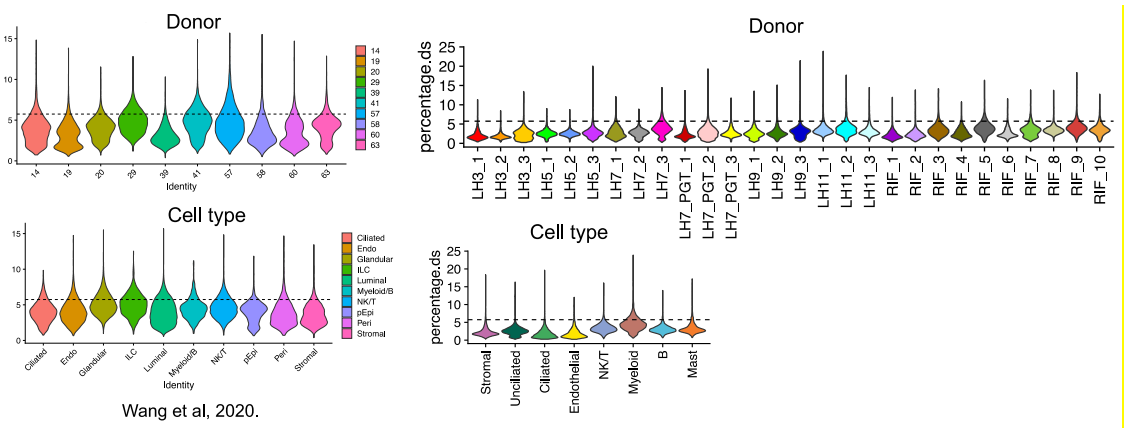
Response: We totally agree that sample pre-processing is fundamental to generate high-quality single cell sequencing data. We have summarized in the table below the dissociation protocols used in published single-cell endometrial studies. The first 4 studies used a total enzyme dispersion duration of at least 60 min at 37°C. Study 5 used overnight collagenase treatment at a low temperature and a short (20 min) TrypLE Select dispersion at 37°C. A shorter enzyme treatment protocol was used in the last two studies. Overall, our collagenase treatment at 37°C for 1 hour was similar with majority of the reported studies.

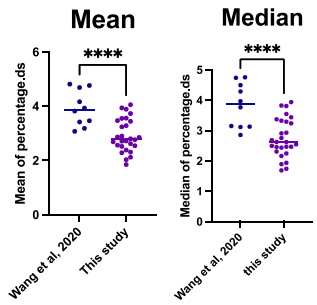
Table. Single-cell dissociation protocols for endometrium in published studies

#	Publication	Journal	Dissociation protocol	Remarks
1	Lucas et al, 2020.	Communications Biology	Collagenase Type IA, 37 °C, 60 min	Endometrium
2	Lv et al, 2022.	PNAS	1. Trypsin, 37 °C, 8 min 2. Collagenase Type I, 37 °C, 60 min	Thin and control endometrium
3	Fonseca et al, 2023.	Nature Genetics	Collagenase, 37 °C, 90 min	Endometrium and endometriosis

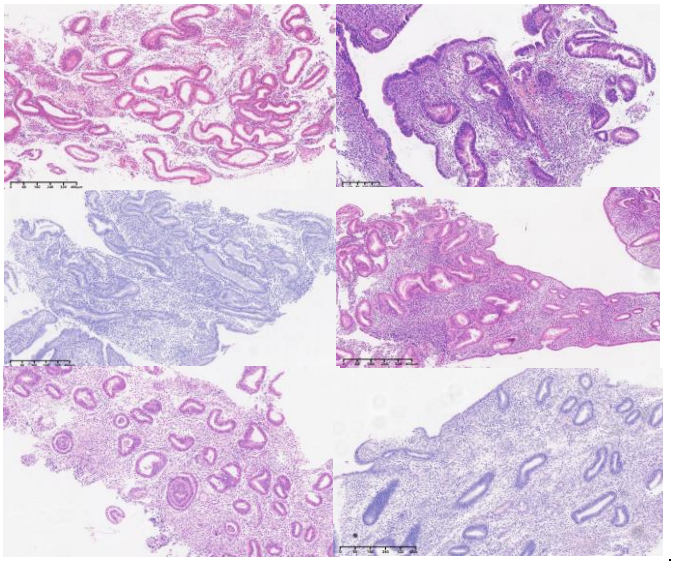
4	Garcia-Alonso et al, 2021.	Nature Genetics	1. Collagenase V, 37 °C, 45 min 2. Trypsin-EDTA, 37 °C, 20 min	Endometrium
5	Wang et al, 2020.	Nature Medicine	1. Collagenase A1, 4°C, overnight 2. TrypLE Select, 37 °C, 20 min	Endometrium
6	Lai et al, 2022.	Theranostics	Collagenase type IV, 37 °C, 15-20 min	RIF and control endometrium
7	Tan et al, 2022.	Nature Cell Biology	Miltenyi gentle MACS Dissociator, 1 min, 20-40 min, 15 min (combine single cells from each stage of a serial digestion)	Endometrium and endometriosis

We have also used a published *in silico* analysis approach [34] to determine the percentage of reads that map to dissociation-affected genes (percentage.ds) in both our dataset and a published human endometrium dataset (study 5, Wang et al., 2020). The mean/median percentage.ds was significantly lower in our study than that of Wang and coworkers (2020) (figure below), suggesting less overall dissociation-effect caused by the protocol used in our study. Importantly, all the biopsies were processed by the same technician (J.W.) with standard procedures which might be unlikely to cause bias finding in a time-series setting.





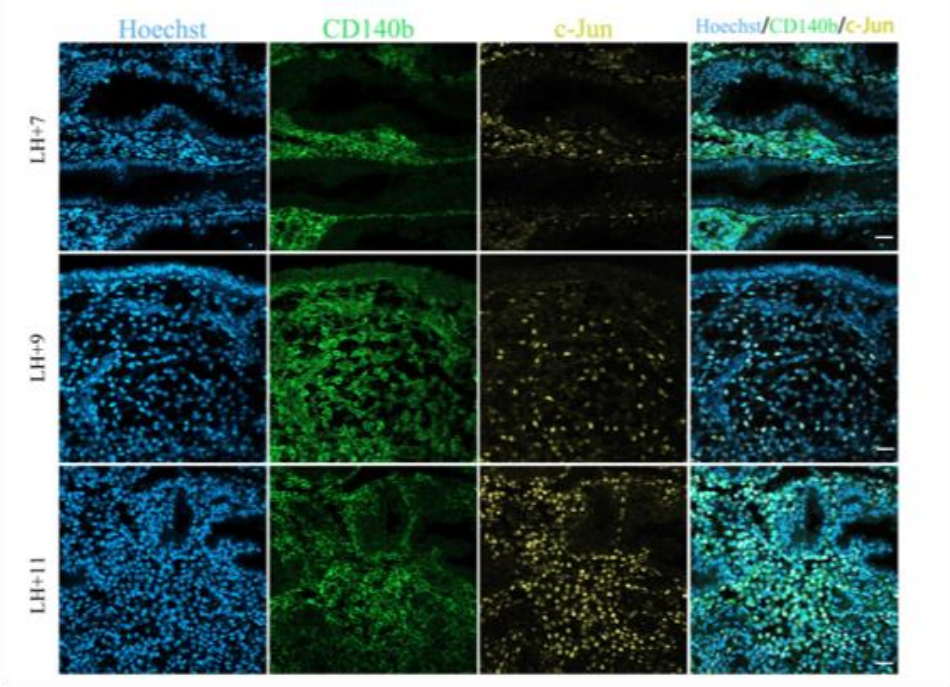
Below we showed example H&E images from our samples. Tissue difference/variation could be seen which is in line with the data in this study.



R2.2-Extensive and stringent digestion can impact drastically the transcriptomic signature too. In this case where authors chose a long digestion, it is particularly concerning since some of the key markers for cells populations are related to stress response-induced genes such as JUN/FOS like in stromal cells (Str3) or immune cells (NK, T cells in the StemVAE analysis). As well, DEG lists show the presence of mitochondrial genes and unannotated sequences or LncRNA within the top DEG. Can the author analyze the dissociation-induced stress response across different cell types/subtypes?

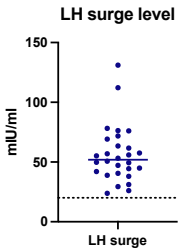
Response: From a biological view, preparation for implantation is a physiological stress to the endometrium. One of the preparatory events in humans is decidualization under the action of progesterone independent of an implanting embryo. During decidualization. apart from morphological transformation (Fig 3), the human endometrial stromal cells also exhibit endoplasmic reticulum stress response, unfolded protein response, and increase their production of proinflammatory factors [35]. In addition, the decidualized stromal cells express high amount of antioxidant enzymes, such as superoxide dismutase [36], and are resistance to oxidative cell death [37]. In this regard, the mid-secretory phase endometrium produce Serum- and glucocorticoid-inducible kinase-1 (SGK1) essential for the induction of anti-oxidant enzymes. A deficiency of SGK1 increases susceptibility of human decidualizing stromal cells to oxidative cell death and is associated with recurrent pregnancy loss [38]. Interestingly, through comparison analysis, 68 out of 140 dissociation-affected genes [34] were also dramatically changed during *in vitro* decidualization [39], indicating their faithful representation of intrinsic cellular processes rather than simply dissociation-effects. Additional supportive evidence included dynamic expression of the stress-related genes such as JUN/FOS during WOI revealed by independent methods[40], functional involvement of JUN and FOSL2 during decidualization[39] and their regulation by the progesterone receptor PGR[39,41]. Moreover, JUND and JUNB were also

decidualization related [42,43]. The stress-related FOS/JUN pathway regulates inflammation [44] which is a feature process during and after WOI. To further validate the expression of stress-gene before tissue dissociation, we performed immunostaining of c-Jun and found its wide expression in stromal cells of mid-secretory endometrium (figure below). Altogether, the stress-related genes identified here were dynamically expressed and correlated with decidualization and inflammation, rather than a dissociation artifact.



R2.3- The analysis of the endometrium during the WOI is very interesting. However little information is provided around LH peak and sampling of the biopsies. Did the authors check LH levels at LH+1, +3,… to verify that all samples biopsies were collected at similar times related to the LH peak/individual? Were all LH peaks similar across patients?

Response: In this study, we determined serum LH, E_2 and P_4 levels at around the expectant ovulation day which started when a dominating follicle of size >14mm was observed with ultrasonography. We determined serum LH instead of urine LH to ensure accuracy in timing the ovulation event. We also looked for a decreasing E_2 and an increasing P_4 levels, which were complimentary indicators for LH surge. The LH surge levels for all samples are depicted in the figure below. Women with a LH level above 20 mIU/ml (dashed line) was considered having a LH surge. There were individual heterogeneity in the LH surge level (mean: 56.25; range: 23.77 to 131 mIU/ml), consistent with a previous study reporting large individual variation in LH surge level (range: 12.1-104.0) [45]. Once the surge day was determined, LH+3, LH+5, LH+7, LH+9, and LH+11 was scheduled for endometrial biopsy by simple day counting without serum LH detection.



For the endometrial biopsy, a Pipelle catheter was placed to the uterine fundus under the guidance of abdominal ultrasound. Then, suction was performed along with downward movement to the internal cervical ostium. Biopsy of all subjects was done by the same doctor according to our standard operation procedure to minimize variation.

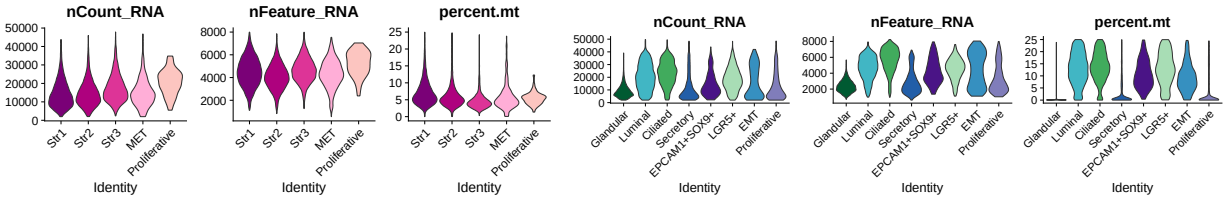
The LH surge determination and endometrial biopsy was described in the Methods (Tissue collection) but largely modified with details as suggested (line 701-709).

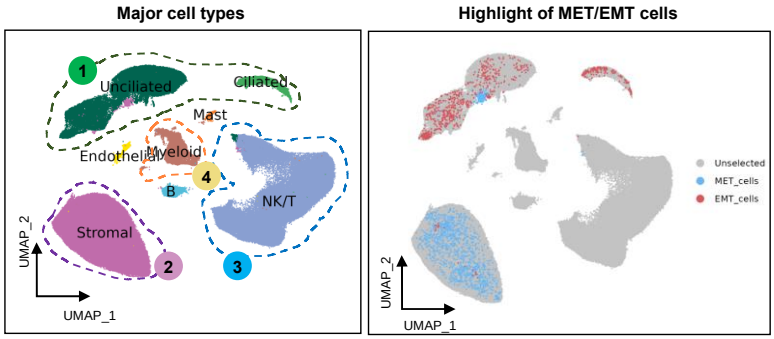
R2.4- Cell type annotations EMT/MET: I find concerning that the authors claim the presence of EMT/MET cells without any further validation (IHC/IF at least should be used to confirm the existence of these cells in the tissue). How did the author discard the presence of doublets and potential mislabeling of these cells? Particularly, can the authors plot the EMT/MET cells back onto the main clustering? In the provided UMAP, a stromal (pink colored) subpopulation seem to cluster very closely from epithelial cells- is this the MET cluster?

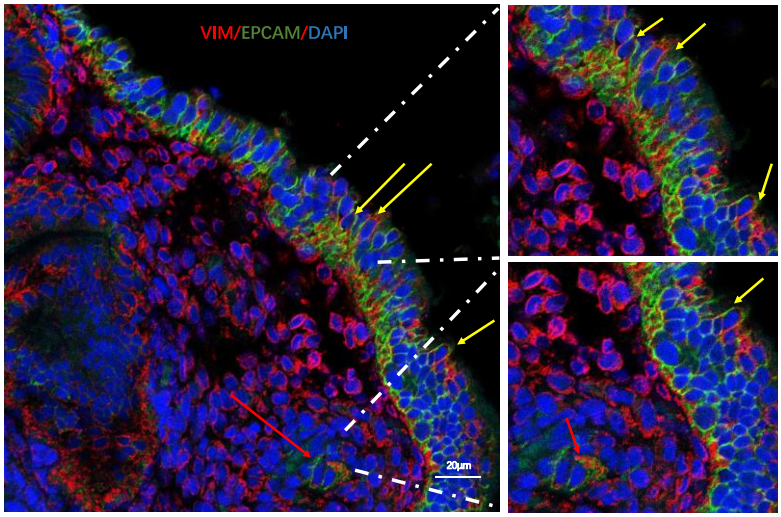
Response: Doublet removal was conducted with DoubletFinder. After doublet removal, we additionally performed iterative clustering and discarded the small clusters (<100 cells) with high number of total UMI, total features and percentage of mitochondrial reads. The EMT/MET clusters identified here did not exhibit high total UMI, total features and percentage of mitochondrial reads compared to the remaining clusters (figure below, top panel), supporting their singlet identity.

As suggested, by plotting back the MET cluster to the major UMAP, it was found that some MET cells (roughly 402 out of 1574, 25.5%,) were in the stromal cells cluster close to the unciliated epithelial cells (figure below, middle panel). Majority of the MET cells were mostly from the main stromal cluster, while the EMT cells were mainly from the epithelial clusters. Moreover, when we retrieved the top 5 neighbors of those MET cells that were close to the unciliated epithelial cells, they were mostly stromal cells (1874 out of 2010 neighbors were stromal cells), indicating their good connections to stromal cells although they are located closer to epithelial cells in the UMAP plot. Therefore, they should not be mis-labeled. In addition, EMT/MET are reported important processes during implantation [46]. Our further immunostaining results showed co-expression of epithelial cell marker and stromal cell marker within the same cell (bottom panel).

We modified the Methods in terms of doublet removal and clustering (line 754-755, line 762-764). The positive immunostaining figure were added in Figure 1 and properly mentioned in the Results (line 187-189).



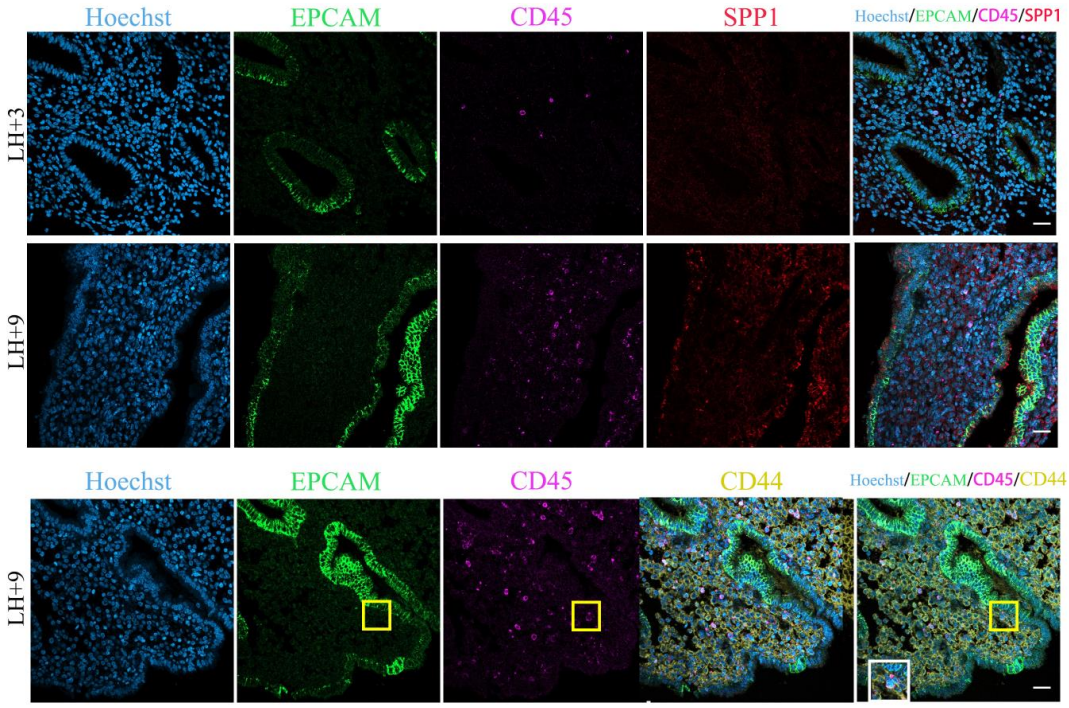




R2.5- The integration of spatial transcriptomics (Visium), albeit at a resolution different from single-cell analysis, was attempted using data from a single experiment and endometrial biopsy from a published manuscript, likely not corresponding to the time points analyzed in this study. I would suggest to validate cell populations, markers and CCC with a spatial transcriptomics/proteomic assay, single cell resolution if possible, using comparable samples and justifiable n.

Response: Thank you for the suggestion. To solve the limitation of scRNA-seq in identification of CCC without spatial information, we utilized a public endometrial spatial transcriptomic (ST) dataset. Although

this dataset did not exactly correspond to the timepoints in this study, the secretory phase samples of the dataset were helpful to reduce some false positive CCCs in our analyses. To clarify, we only used this ST dataset to help candidate the strong ligand-receptor pairs with spatial proximity in our own data. Therefore, the strategy of integrating ST data did not introduce false discoveries. We also additionally performed some validation analyses. Firstly, endometrial tissue immunostaining experiments of epithelial cell marker EPCAM and stromal cell marker VIM showed co-expression of these two markers in one cell indicating the presence of EMT/MET subpopulation (**see response to R2.4**). Secondly, Immunostaining of stress-related marker c-Jun in endometrial tissue before dissociation showed a wide expression of c-Jun in the stromal compartment implicating their physiological role in decidualization rather than a dissociation artifact (**see response to R2.2**). Thirdly, regarding the CCCs between SPP1 and CD44, immunostaining of SPP1 in endometrial tissue from LH+3 and LH+9 validated their high expression after WOI opening. Moreover, proximal location of CD44-expressing $CD45^{+}$ immune cells to the epithelial cells were also demonstrated (Figure below, added as Supplementary Figure 6). We hope in the future work, a more comprehensive characterization strategy could be done to the endometrial biopsy from relevant timepoints.



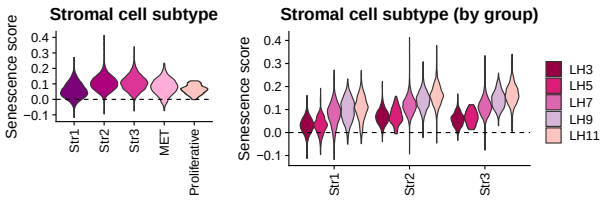
R2.6- CCC analysis: are the L/R interactive pairs validated across all patients?

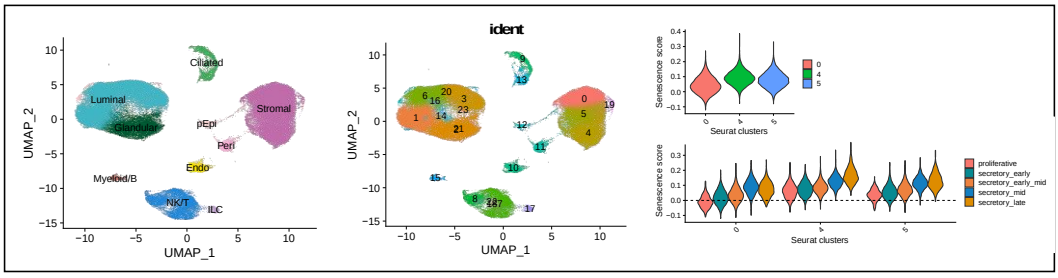
Response: For the CCC analyses, the identified L/R pairs were based on data of the normal fertile population. As suggested, we have performed the analysis on RIF endometria and compared between RIF and control endometria. The results show differential interactions between the two groups for some of the identified

L/R pairs. The results are summarized in Supplementary Figure 11 and discussed (line 659-661) in the revised manuscript.

R2.7- Senescence: authors combined all cell subpopulations within a specific cell type to evaluate the putative senescence signature during the WOI - using SenMAyo, a senescence signature derived from human/mouse adipose tissue and bone marrow (not validated in teh endometrium). Does this apply to all stromal cell clusters? Surely there are key differences between stomal cell subpopulations, but do they all play a similar role in WOI and display similar senescence markers? Orthogonal validation would be good to confirm any of these claims

Response: Based on this comment, we have validated the SenMayo gene set in human endometrium and confirm their ability to indicate endometrial ageing/senescence (Supplementary Figure 5 in the revised manuscript). Next, we have calculated the senescence score for the identified stromal subpopulations. They all demonstrate a positive senescence score (figure below, top panel). Moreover, the main subpopulations exhibit a dynamic changing pattern across the WOI, consistent with the results in Extended Fig. 7a,b which shows a similar changing pattern of expression and a high overlap of dynamic genes among different subpopulations. Orthogonal validation was done in an external dataset GSE111976 which studied endometrium across the menstrual cycle [12]. The results also showed comparable senescence score across main stromal cell clusters (figure blow, bottom panel) as well as similar increasing pattern across the phases. Other than epithelial cells with well-defined subtypes (luminal / glandular / ciliated / secretory), the biological function/implication of stromal cells subpopulations is largely unknown. Therefore, we combined all the stromal cells to study their dynamic characteristics during the WOI in this study. This strategy was also adopted by the study we mentioned [12].



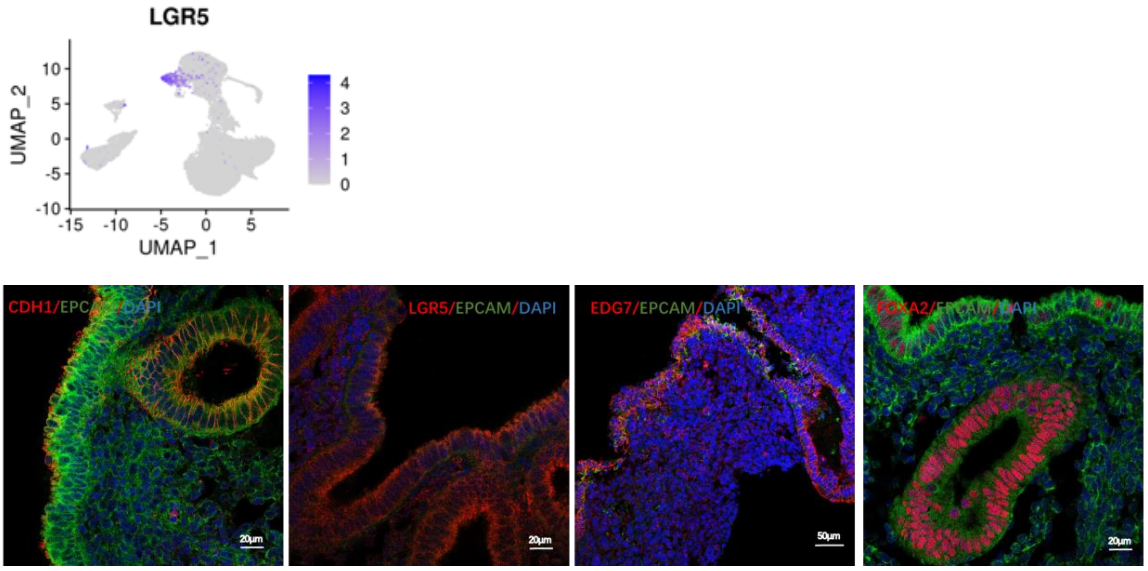


R2.8- I would suggest to refrain from statements such as "Altogether, the continuous elevation of the senescence score from LH+7 supported that the stromal senescence tunes the endometrial inflammatory microenvironment." At best, the analysis shows association, and without any orthogonal validation or functional validation/mechanistic study, this looks like an overstatement. Same for sentence line 298- authors have shown association, but not correlation.

Response: Thank you very much for this critical comment. The statements were modified to avoid overstatement/misunderstanding as suggested. Also, careful inspection and proper modification were performed throughout the whole manuscript.

R2.9- LGR5+ cells: authors suggest LGR5 is expressed in luminal/progenitor epithelial cells, but gene expression data shows no expression of LGR5 in the luminal compartment (Ext fig 2g). Additionally, the IF image (fig 1) suggest that LGR5 is also present in stromal cells, in addition to epithelial cells. Are those luminal or glandular cells? can you provide further details about RNA/protein detection of LGR5 within endometrial cells?

Response: The cited publications used LGR5 as a marker to identify luminal epithelial cells and epithelial cell progenitors. The $LGR5^+$ epithelial progenitors mostly reside in the luminal compartment. In this study, a $LGR5^+$ progenitor population was identified. The fact that minimal LGR5 expression in the luminal compartment was observed in Ext fig 1e may be attributed to the limitation of single-cell transcriptomic assay. However, protein expression of LGR5 was detected in the luminal and glandular epithelial cells in endometrium of proliferative phase during which glands are newly formed [47,48]. During this phase, new glands are being formed which sometimes are hard to discern from stromal cells. Additional labels have been added to Fig 1 to clarify that the positive LGR5 was not in stromal cells. Moreover, additional immunostaining of LGR5 was performed in independent samples and the results showed the positive staining of LGR5 in epithelial cells (figure below, added in Supplementary Figure 1 in the revised manuscript).



R2.10- Extended Fig 3d: how stable is this tree and subjected to variable cell numbers for each category? What do the top numbers correspond to?

Response: The tree (Extended Fig 3c) was generated by comparing the cell relationship in the clusters identified from different parameter setting. Different rows displayed the cluster information with different parameters (cluster resolution). For example, on the top row, the top numbers correspond to 9 clusters identified with a clustering resolution of 0.2, while on the bottom row, the numbers correspond to 37 clusters identified with a clustering resolution of 3. We could know from the tree how cell clusters in a high resolution go into the next cluster in a low resolution. Given the clustering is deterministic, the tree is a direct readout and should be considered as stable. This tree helps better cell annotation.

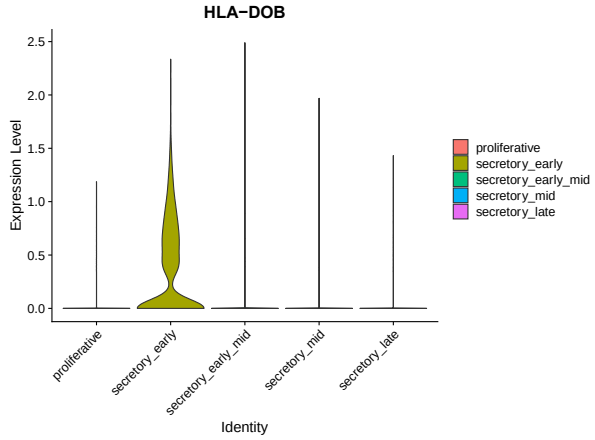
R2.11- Line 341- CDH1 is defined as a cell-cell adhesion molecule specific to epithelial cells and its loss has been shown to induced epithelial cell disorganization and as a consequence is linked to infertility in mouse studies. Can authors provide human-based literature to show that it is a "known" regulator of implantation?

Response: In addition to the cited mouse study, an in vitro study demonstrated that the endometrial receptivity marker HOXA10 directly upregulated the expression of CDH1 in human endometrial epithelial cells thereby affecting trophoblast spheroid (embryo surrogate) attachment (Bi et al, 2022) [49]. The information has been added in the revised manuscript (line 400, highlighted).

R2.12- Can the authors confirm the expression of HLA-DOB in epithelial cells through orthogonal validation?

Response: We have checked the expression of HLA-DOB in a published single-cell dataset of endometrium with histological phasing information. The results show that HLA-DOB is expressed in the early secretory phase consistent with our finding. We then used 4 samples of primary endometrial epithelial cells and 2 endometrial epithelial cell lines for qPCR using published primers (F:GTGGCTCTGCTAGTGAATCTGAC; R:GTCCCGTTGGTGAAGTAACAGTC) of HLA-DOB. The results are shown in the Table below. According to the Ct values, HLA-DOB is detectable in the primary endometrial epithelial cells from both proliferative and early secretory phases, but undetectable in the cell lines.

HLA-DOB expression in single-cell dataset (GSE111976)



qPCR results of HLA-DOB in human endometrial epithelial cells

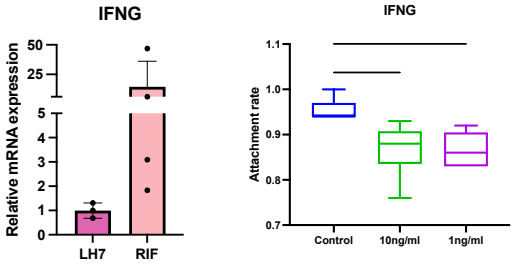
Sample information			Ct value of GAPDH	Ct value of HLA-DOB
Fresh isolated epithelial cells from endometrial biopsies	Sample 1	Proliferative	23.42	27.49
	Sample 2	Proliferative	20.5	26.01
	Sample 3	Secretory	22.78	29.89
	Sample 4	Secretory	23.34	28.13
Endometrial epithelial cell lines	HEC1-B		21.07	36.85
	Ishikawa		24.26	Undetermined

R2.13- The computational analysis suggest or predicts but without any validation (functional/orthogonal) this is not a demonstration (line 413). This type of statement comes through often in this manuscript and I would suggest to carefully use such statement when driven by computational inference only.

Response: As suggested, ‘demonstrated’ was changed to ‘exhibited’. Meanwhile, rewording was performed throughout the whole manuscript to avoid such statement.

R2.14- While author conduct a thoughtful computational analysis of the immune/myeloid cells within RIF samples based on the obtained single cell data, they do not provide any functional validation/orthogonal validation to sustain the claims of correlation or hyperinflammation, nor the implication in impaired receptivity for these particular cell types.

Response: As IFNG is highly expressed in immune cells, qPCR of the total RNA showed high IFNG production in the RIF samples (figure below, left panel). In addition, we confirmed the presence of IFNGR in the receptive epithelial cell line ISHIKAWA. Further attachment assay showed that addition of IFN-gamma protein (cat no. HY-P7025) to ISHIKAWA reduced the attachment rate of the trophoblast spheroid (figure below, right panel). This result was added to Supplementary Figure 10 in the revised manuscript.



Reviewer #3 (Remarks to the Author):

This manuscript reports on the uterine endometrial transcriptome at single cell resolution across the secretory phase of the menstrual cycle in fertile volunteers (controls) and in the window of implantation (WOI) in cases with a history of recurrent implantation failure (RIF). The data from controls add to recent datasets with novelties being that samples were obtained timed to the LH surge before, during, and after the WOI, and application of an algorithm, StemVAE, developed by the authors to model time-dependent endometrial single cell data across the secretory phase. Several key findings in controls without RIF include interpretation of the data that reflects a gradual transition of epithelial cell gene dynamics across the WOI, in contrast to an abrupt transition of stromal cells at LH+7 (the opening of the WOI) and minimal changes in other cell types; identifying luminal-glandular characteristics of luminal epithelial cells; identifying a set of epithelial receptivity genes, identifying epithelial and stromal cell cross-communication with immune cells, limited inflammatory features in the WOI and more robust inflammation beyond the WOI (consistent with the tissue preparing for menstrual shedding), and that senescent stromal cells may control inflammation. The RIF WOI samples were classified as "displaced" or "dysregulated" based on differences mainly in epithelial cluster profiles and especially immune cells that were characterized by a hyper-inflammatory environment in the "dysregulated" phenotype, compared to controls. Overall, the manuscript provides a valuable dataset of endometrial cell dynamics across the secretory phase of the menstrual cycle. However, several items warrant further clarification to assure rigor of the results, interpretation of some of the data, and to support some of the conclusions drawn.

Specific Comments to be addressed:

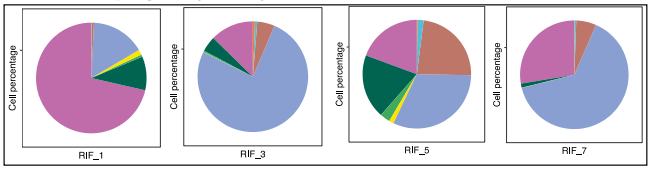
R3.1. While cases and controls were fairly well-matched by BMI and age, it is important to include in Supplemental Data 1 the gravidity and parity of the donors (i.e., how many pregnancies, miscarriages, live births each had). Prior pregnancy may impact endometrium in various ways, and so this needs to be considered and evaluated in terms of the marked heterogeneity of the data among controls and in differences between RIF versus controls (see below).

Response: As suggested, gravidity, parity, miscarriage/abortion, and implantation failure have been added to Supplementary Table 1，partially displayed below. We would like to mention that the 2 abortions for one fertile LH7 donor (LH7_PGT_3) were induced abortion because the fetuses carried compound heterozygous mutations for beta-thalassemia. This donor then received PGT-M and got pregnant after single-embryo transfer in a later cycle following the endometrial biopsy for this study. Among the cohort in this study, only 1 fertile donor (LH7_PGT_1) had no pregnancy history at the time of biopsy while the other fertile controls have comparable history with 1 to 2 pregnancy or live birth at the time of endometrial biopsy. Thus, the cellular heterogeneity in the control population should not be caused by

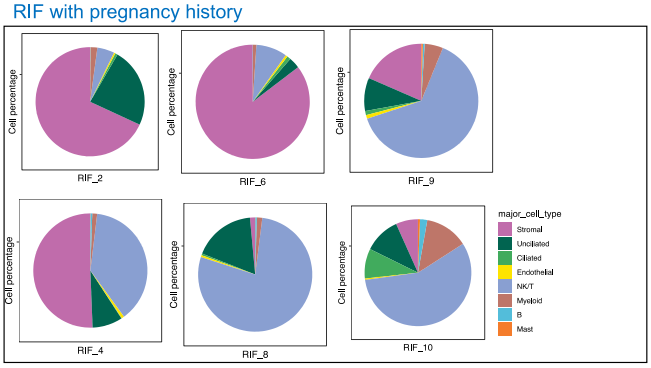
differences in pregnancy history. Moreover, the stemVAE, which was built on the control donors with pregnancy history, can correctly predict the time domain of the fertile donor without a pregnancy history to be at LH+7 (Extended fig. 5b), implying that pregnancy history is unlikely to affect the identification of WOI in our settings. For the RIF donors which were recruited under RIF definition [50], 4 out of 10 had no pregnancy history while the remaining 6 had pregnancy history. The 6 with pregnancy history was diagnosed with secondary infertility more than 1 year apart from their last pregnancy. No obvious association was found between pregnancy history and cellular composition (figure below). Moreover, the numbers of implantation failure were comparable between RIF donors with and without a pregnancy history (P value=0.1458). These observations show that the sample setting of the study can characterize the physiological and pathological changes of transcriptomes in the WOI and that the changes are unrelated to the pregnancy history of the donors.

Original Donor ID	Sample ID	Times of gravidity	Times of parity	Times of miscarriages /abortion	Times of implantation failure	Remarks
E23	LH3_1	2	2	0	-	
E24	LH3_2	1	1	0	-	
E25	LH3_3	1	1	0	-	
E12	LH5_1	2	2	0	-	
E13	LH5_2	1	1	0	-	
E20	LH5_3	1	1	0	-	
E08	LH7_1	1	1	0	-	
E11	LH7_2	1	1	0	-	
E14	LH7_3	1	1	0	-	
E03	LH7_PGT_1	0	0	0	0	
E04	LH7_PGT_2	1	1	0	0	
E09	LH7_PGT_3	2	0	2	0	Induced abortion due to compound heterozygous mutations for beta-thalassemia in fetus
E15	LH9_1	1	1	0	-	
E19	LH9_2	1	1	0	-	
E21	LH9_3	1	1	0	-	
E22	LH11_1	1	1	0	-	
E27	LH11_2	1	1	0	-	
E29	LH11_3	2	2	0	-	
E01	RIF_1	0	0	0	4	
E07	RIF_2	2	0	2	4	Secondary infertility
E18	RIF_3	0	0	0	3	
E28	RIF_4	4	1	3	3	Secondary infertility
E30	RIF_5	0	0	0	5	
E02	RIF_6	3	1	2	3	Secondary infertility
E05	RIF_7	0	0	0	4	
E31	RIF_8	1	1	0	4	Secondary infertility
E32	RIF_9	2	0	2	3	Secondary infertility
E33	RIF_10	4	0	4	3	Secondary infertility

RIF without pregnancy history



RIF with pregnancy history



R3.2. For the RIF patients, were the endometrial biopsies evaluated for endometritis, a common cause of RIF? This would be to document if CD138+ plasma cells were found in the endometrium, and if so were patients treated? Was this also evaluated in controls? This is essential to perform in the RIF samples, as the data demonstrated intense endometrial inflammation in the RIF WOI. Where might this come from, as some disorders predisposing to endometrial inflammation (e.g., infection, endometriosis and other) were apparently among exclusion criteria for donors?

Response: Both the RIF and the fertile donors were evaluated for $CD138^+$ cells at the time of endometrial biopsy. The $CD138^+$ plasma cells were detected only in 3 samples; 1 RIFClass1 sample showed 1 $CD138^+$ plasma cell per high power field (HPF), while 2 fertile samples exhibited 2 and 6 $CD138^+$ plasma cells per HPF. For the latter fertile sample, the 6 $CD138^+$ plasma cells were observed only in one HPF but not in other areas of the sample and was likely to be an incidental observation. Currently, there is no consensus on diagnostic criterion of chronic endometritis (CE) based on the $CD138^+$ plasma cells. Referring to a publication which established a fertile reference for CE diagnosis as having $\geqslant$5.15 $CD138^+$ plasma cells/0.1 mm^2 [51], we considered all our samples were negative for CE. Most importantly, the 3 samples with $CD138^+$ plasma cells detected were NOT in the RIFclass2 group which the data showed endometrial inflammation. All the donors did not receive any antibiotic treatment in the recent 3 months prior to biopsy. The above information has been described in the Methods section in the revised manuscript (line 697, 720-721).

	# of $CD138^+$ plasma cells / HPF	# of $CD138^+$ plasma cells / 0.1 mm^2
PGT-fertile donor (E03)	6	2.27
Fertile donor (E20)	2	0.76
RIF_8 (E31)	1	0.38

Regarding the origin of the inflammation if infection and endometrial inflammatory diseases are precluded, we suspect the inflammatory status is induced by an intrinsic cellular mediation under the actions of hormones. A pro-inflammation environment has long been reported to be necessary for embryo implantation [52,53]. Consistently, we found dynamic changes of inflammation score during stromal cell decidualization. The high inflammation status of the RIF endometrium without evidence of infection might indicate dysregulation of the intrinsic cellular programs or responses to hormonal actions.

R3.3. How do the authors know donors and RIF volunteers did not have endometriosis, adenomyosis, endosalpingiosis, hypdrosalpinx, or other conditions that are associated with an inflammatory milieu in the endometrium – especially in RIF cases and also in controls, which were mainly "public". Did they have imaging and/or surgery to assess these conditions that affect the endometrium? [Clarification]

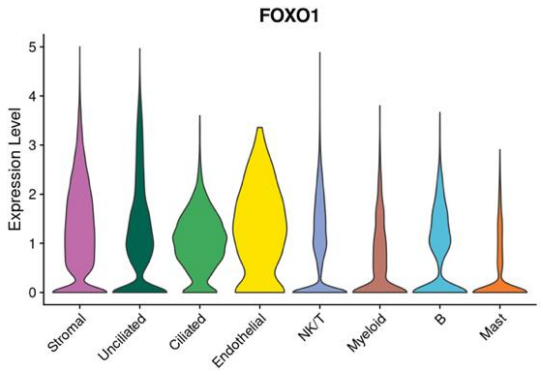
Response: In this study, the recruited donors received vaginal ultrasound examination to exclude endometriosis, adenomyosis, endosalpingiosis, hypdrosalpinx and so on. The donors also reported no pain or other related clinical manifestations. Therefore, we assumed that they were free of endometriosis without further laparoscopic confirmation, an unjustifiable procedure to the fertile donors. In addition, we conducted $CD138^{+}$ plasma cell assay for all donors to exclude CE (response to **R3.2)**.

R3.4. Figure 1b: transcriptomic dimensional reduction revealed epithelial luminal, secretory, glandular, and proliferative subpopulations. To what does the proliferating population refer – proliferating, cycling cells? And the secretory cells - are those luminal, glandular, and all unciliated? Please clarify.

Response: In Figure 1b, the proliferative population refers to the cell cluster that highly expressed the proliferating marker MKI67 and TOP2A, while the secretory cells refer to those that highly expressed the secretory markers PAEP, GPX3, SPP1. The latter cluster of cells also expressed luminal and glandular markers indicating they were unciliated epithelial cells. These information have been clarified in the revised manuscript (line 150).

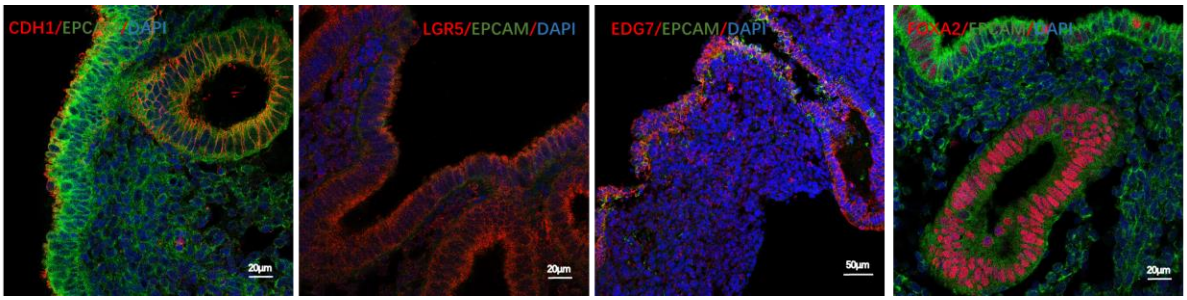
R3.5. Figure 1c: it is surprising that epithelial cell markers do not include FOXO1, as it is a major contributor to epithelial integrity and is a core transcription factor of epithelium involved in endometrial receptivity. Please explain.

Response: The cell annotation markers with cell type-specificity were mostly collected from previous single-cell endometrium studies and other relevant studies. FOXO1 is an important transcription factor not only for endometrial epithelium but also for stromal cell decidualization. The gene was found to be a potential core regulator in subsequent analyses (Figure 3&4) for both the epithelial cells and the stromal cells. As the gene is not epithelial cell-specific (figure below), it is therefore not included in Figure 1c which shows the cell identity specific marker expression.



R3.6. Figure 1e: the validation is done in proliferative endometrium (before the LH surge). It should be in secretory endometrium (when the samples were obtained for scRNA seq).

Response: We chose proliferative endometrium in Figure 1e because new glands were formed mostly in the proliferative phase [54], which should be easier to test the hypothesis that the luminal cells can form glands. But as suggested, we repeated the experiment on secretory endometrium. The results are similar to that of the proliferative endometrium (figure below). The result has been added to Supplementary Figure 1 and mentioned in the Results section in the revised manuscript (line 172-174).



R3.7. Extended data Fig 2. The extensive inter-individual abundance of various cell types across all samples is attributed by the authors to intrinsic differences in the various tissues. Is this reproducible from cycle to cycle within the same donor? Also, the vast heterogeneity could also be due to prior history of pregnancy or different hormonal milieu at a given time point post LH surge. Having serum estradiol and progesterone levels are key to determine if hormone levels could contribute to this heterogeneity in this highly steroid hormone-responsive tissue and its constituent cells, in addition to histology of the tissues at given time point. Also, different regions of the endometrium being sampled for analysis could account for this heterogeneity – e.g., is an epithelial cell adjacent to an immune cell the same as if it were near a fibroblast?

Response: We agree that the observed tissue heterogeneity can have several possible causes:

a. Variations in tissue region and amount. In the study, we controlled the variation by standardizing the biopsy procedure and assigning the same doctor to collect the tissue. Nevertheless, variations might still exist due to differences in endometrial thickness and tissue texture. There was one donor who received biopsy twice at LH+7 in two menstrual cycles one year apart. StemVAE determined that the two samples were both at LH+7 because of their similar molecular expression. However, the cellular compositions of these two biopsies were different (table below) showing that tissue heterogeneity did not markedly affect bioinformatics analyses.

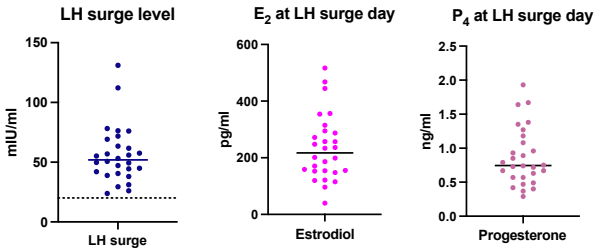
   Cell composition of the same donor in two menstrual cycles.

	Stromal	Unciliated	Ciliated	Endothelial	NK/T	Myeloid	B	Mast

Biopsy 1	0.83677	0.0820	0.0013	0.0020	0.0706	0.0073	0.0002	0.0003
Biopsy 2	0.0490	0.2541	0.0903	0.0046	0.5302	0.0656	0.0062	0.00

b. Reproductive history. It is well established that pregnancy imposes stress to the mothers. Whether cessation of pregnancy will reverse the effects is unknown though it is recently reported that pregnancy cessation reversed maternal biological aging [55]. There is no report on the effect of pregnancy on endometrium. This might be due to difficulty in collecting endometrial tissue from the same women before and after pregnancy. Nevertheless, there were studies comparing IVF outcome for patients with different pregnancy history. Earlier studies reported conflicting results on the prognostic value of an early pregnancy loss after IVF-ET: Levy et al reported better ongoing-pregnancy rate in women with preclinical abortions[56]; whereas Acosta et al reported worser ongoing-pregnancy rate in the preclinical abortions group[57]. Two recent studies with a large sample size (>25000) reported no association of previous eutopic pregnancy with IVF outcome such as 'live birth rate' [58,59]. In this study, as stated in the response to **R3.1**, reproductive history of the fertile group was maximumly controlled and showed no evidence of affecting the WOI. In the RIF group, the reproductive history was not associated with cellular heterogeneity. These results, together with previous findings that women seeking IVF treatment with or without a previous reproductive history (pregnancy loss, termination of pregnancy and live birth) had no significant effect on IVF outcome [58], suggested that once the patients are diagnosed as infertility, the pregnancy history is unlikely associated with current endometrial receptivity.

c. Hormone levels. Endometrium is hormonal sensitive. Variations in estrogen (E_2), progesterone (P_4), and LH levels exist among individuals. In this study, we noticed individual heterogeneity in the LH/E_2/P_4 levels on LH surge day (figure below). The variation of LH surge level (mean: 56.25; range: 23.77 to 131 mIU/ml) was consistent with a previous report (range: 12.1-104.0) [45]. We agree with the important role of circulating hormones in tissue heterogeneity of the endometrium. Unfortunately, we did not collect donors' serum at the time of endometrial biopsy hindering analysis between hormone level and tissue heterogeneity. However, the serum P_4 level may not correlate with the level of endometrial P_4, which was essential for endometrial receptivity [60]. Future receptivity-related studies should include determination of the hormones in the circulation and samples at the time of endometrial biopsy.



R3.8. Figure 2b needs more explanation in the legend and some labeling in the figure per se.

Response: The legend of Figure 2b has been modified, and additional labels have been added to the figure in the revised manuscript.

R3.9. Figure 2d: M&M says n=3 from each group at LH+3, LH+5, LH+9, LH+11 and n=10 from LH+7. The group ratios (different colored bars) don't reflect this at the bottom of the panel (and in other figures, too). Please explain.

Response: There are several reasons for the non-proportional cell number in Figure 2d. First, the figure shows data characteristics of the fertile population only (n=10) at LH+7 and the RIF samples were excluded. Second, as mentioned in the first part of the Results section, the cell percentage varied a lot across samples in the same collection timepoint which meant different cell abundance in terms of epithelial cells, stromal cells, and immune cells in each sample. Figure 2d display epithelial cells and stromal cells separately. Therefore, the group bar did not have a comparable ratio.

R3.10. How did Stem-VAE account for differences in cell abundances for all the samples?

Response: During development, we tested the performance of StemVAE in different settings including gene set, cell number, sequencing depth. For cell number, StemVAE was stable irrespective of sampling same/small number of cells (500/donor, 1000/donor, 2000/donor) from each sample, or sampling equal cell number for each cell type (table below). Therefore, the StemVAE is already robust to the cell abundance.

	Spearman's rank correlation coefficient	Number of cells subset
Stromal	0.86683	4000
Unciliated	0.81938	4000
Ciliated	0.39617	3053
Endothelial	0.52899	606
NK/T	0.48732	4000
Myeloid	0.29056	3723
B	0.07789	1714
Mast	-0.13112	1127

R3.11. Stem-VAE revealed dynamic changes of the epithelial cells and stromal cells that highly correlated with time across the WOI, but the immune cell features appeared to be time-invariant. This is

in stark contrast to histologic data of immune populations changing across the cycle, and especially beyond the WOI when the tissue prepares for shedding, which the authors also found. This seems inconsistent. Please explain.

Response: The time-invariant property of the immune cells is a relative concept when compared to that of the stromal and epithelial cells, which differentiate/transform under the control of hormones. Figure 2c and Extended Fig. 6a show that the predicted time of the stromal and epithelial cells from StemVAE (a gene-expression based time prediction model) is highly correlated with the biological sampling time, whereas the other cell types exhibit low correlation. The NK/T cells had a correlation coefficient around 0.5, indicating that their molecular changes across the WOI were not that time dependent but were not completely unchanged along the time axis. Extended Fig. 2b shows that the immune cells exhibited some gene expression changes at LH+11 especially for the T cells. Changes in gene expression of the immune cells occurred mostly at LH+11 supporting tissue propensity for shedding at this time point. Moreover, in the CCC analyses, we noted that the immune cell changes were mainly maturational and functional related. These results are consistent with previous findings. Previous histological data showed immune cell infiltration variation across the menstrual cycle and revealed the stark increase in cell number in the mid-/late-secretory phase [61,62]. However, the cell abundance analysis was not the focus of the study because of its large variation with limited sample size. Therefore, further larger studies are still required.

R3.12. While epithelial glandular, luminal, secretory, and EMT subtypes displayed differential time-associated gene expressions, the ciliated, EPCAM1+SOX9+, LGR5+, and proliferative subpopulations did not. What are the "proliferative" subpopulations?

Response: The proliferative subpopulation refers to the cycling epithelial cell cluster. This has been clarified in the revised manuscript (line 343).

R3.13. In Figure 3, stromal fibroblast profiles in LH+3 and LH+5 look very similar, consistent with bulk transcriptomic data by others. Important to cite these supportive bulk RNA seq or microarray studies done by others.

Response: As suggested, we did a comprehensive search and found two publications reporting the LH+3 and LH+5 endometrial transcriptome [63,64]. One of them (Horcajadas et al, 2008) showed expression similarity between LH+3 and LH+5 at bulk transcriptomic level rather than cell type-specific level. The article has been cited in the revised manuscript (line 288-289). The other one considered LH+3 and LH+5 as one pre-receptive group and did not study their similarity.

R3.14. In Figures 3 and 4, as the authors mention, the data differ from those of the Wang et al

endometrial scRNA seq study across the menstrual cycle who found that the epithelial profiles abruptly open the WOI; whereas, the stromal fibroblast transition is not abrupt. Herein, the Figure 4 dendrogram shows that genes for 2 sub-branches of the distal right branch on the x axis are highly expressed in LH+3 and LH+5 and then abruptly diminish thereafter, opening the WOI, although the genes are different than in Wang et al. In Figure 3 the effect is even more dramatic. The authors suggest their sampling timed to the LH surge, not done in the Wang study, may account for these discrepancies. However, there may be other reasons including different patient characteristics (e.g., prior pregnancies or not, different hormonal milieu, different endometrial histology – again another reason to have the latter 2 items in the clinical metadata and do sub-analyses of the scRNA seq data herein. Also, this should be considered in the Discussion section.

Response: Thank you very much for this critical comment. We agree that the patient characteristics might affect the cellular and molecular manifestation of the endometrium. We retrospectively collected the pregnancy history of the recruited donors and RIF patients and analyzed accordingly (refer to **our response to R3.1**). Regrettably, we did not measure the steroid hormone levels at the time of endometrial biopsy. This limitation was added in the Discussion section (line 636-638). Additionally, the suggested possible reasons for the discrepancy between this study and the previous study were also discussed in the revised manuscript (line 613-617).

R3.15. The WOI is generally considered to span LH+7 to LH+10 for embryo attachment and beginning breaching the epithelium into the stromal compartment. How do the authors reconcile that the epithelial dynamics in Fig 4 show that LH+9 variably resembles LH+7 and the 2 sampling points reveal quite different profiles in the stromal population in Fig 3 with these processes? What is the model in terms of known histology and WOI duration which is an approximation but still spans ~ 4 days?

Response: Although the WOI spans 2 to 4-day by histology, several studies determined the optimal WOI for embryo transfer using different technologies. The present study intended to depict the dynamic characteristics of the WOI. Figure 4 shows resemblance of the LH+7 and LH+9 epithelial cells indicating a at least 2-day duration of the WOI. The 2-day model was also used for the epithelial receptivity analysis in the RIF patients. In Fig 3, similarity of some genes and cells could be seen between LH+7 and later time points for the stromal cells. Therefore, our study did not violate the 2~4-day duration of the WOI. Here, we depicted the dynamic changes for a better understanding of the WOI and showed that the endometrial state is not static but is progressively changing in a cell-type specific manner.

R3.16. How do the epithelial receptivity genes correlate with those in bulk datasets (e.g., Altmae 2017),

and the clinical ERA bulk transcriptome test or the immunohistochemical clinical BCL6 test for inflammation or other epithelial markers of receptivity (e.g., beta 3 integrin)?

Response: We have performed intersection analysis between our epithelial receptivity genes and those from ERA and Altmae 2017 which are at bulk-level. 15 of our epithelial receptivity genes are also found in the ERA test and the Altmae 2017 study. They are MT1G, ARG2, DEFB1, BCL6, GBP2, CLDN4, ID4, ANXA2, DYNLT3, NDRG1, MMP26, CRISP3, SLPI, MT2A, GAST, among which BCL6 is found. Other epithelial receptivity markers CDH1 and CD44 are also present in our gene set. However, beta 3 integrin (ITGB3) is not found in any of the three gene sets (this study, ERA, and Altmae 2017). The comparisons have been added to the Supplementary Table 2 in the revised manuscript.

R3.17. scRNA seq data from the 10 RIF samples resulted in classification of 2 groups: Class 1 with "displacement" of the WOI and Class 2 with "dysregulated" WOI. The main driver was epithelial receptivity scores, and Class 2 samples displayed a hyper-inflammatory microenvironment involving myeloid cells with high upregulation of IFN-γ in NK and T cells and IFN-γR1 in luminal epithelial cells. This is a remarkable set of observations, consistent with bulk data studies (should also cite refs from Macklon's group, including PMC4716345).

Response: Thank you for the recognition of our study. As suggested, supportive publications have been properly cited together with additional discussion in the revised manuscript (line 663-664).

R3.18. With regard to the RIF Class 1 "displacement group" – which way is the window displaced – to a pre-receptive or post-receptive profile?

Response: This group was displaced to a post-receptive stage. It has been clarified in the revised manuscript (line 459-460, line 468-469).

R3.19. With regard to "dysregulated" Class 2, the inflammatory profile warrants further investigation about the patients – see Comments 2 and 3 above about infection and inflammatory disorders.

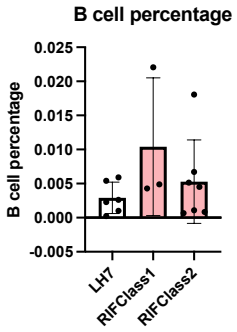
Response: Thank you very much for this critical comment. We have retrospectively investigated this group (Please see our response to **R3.2** and **R3.3**). Briefly, no obvious infection/inflammation has been found in the RIFClass2 patients.

R3.20. Commonly associated with endometrial inflammation is abnormal progesterone signaling in stromal fibroblasts and in epithelium. Did the authors detect abnormal downstream events from PR in Class 2 RIF samples?

Response: Thank you very much for this insightful comment. To answer this question, we first identify the potential PR targets in endometrial epithelial and stromal cells using published results [39,40,65], which reported gene expression changes after PR modulation and PR binding to promoter regions by ChIP-seq. We then compared the DEGs in the RIF epithelial cells and the stromal cells with the PR target genes. There are 6 epithelial DEGs potentially targeted by PR, namely CLDN4/CXCL14/DEFB1/GNLY/GPX3/FAM13A, but no stromal DEGs were potential PR targets. CLDN4/CXCL14/DEFB1/GNLY/GPX3/FAM13A were all reported to be related to inflammation modulation. The information has been added to the revised manuscript (line 636-638).

R3.21. Also, notably in Figure 6a, it appears that there are more B cells in the RIF group(s) – is that correct? If so, the possibility of infection arises. Also, any differences in the B cells between RIF Class I and Class II samples?

Response: Although it appeared that there were more B cells in the RIF group in Figure 6a, the sample numbers were different namely 10 in the RIF group and 6 in the LH7 group. Moreover, the cell percentage analysis exhibited large variation (Extended Fig. 2) across the samples. As discuss above, it is inappropriate to compare cell abundance in the study. In our analysis, the percentage of B cells did not change significant change across the WOI (Extended Fig. 2). No significant difference was observed between RIF and control samples, as well as between RIFClass1 and RIFClass2 (figure below).



R3.22. Other items:

a. When referring to stromal cells, the correct nomenclature is ““stromal fibroblasts”. Please correct throughout the manuscript.

b. Please put the # of RIF samples in the Materials and Methods (only the number of controls is listed there).

c. Figure 1b, central portion – for clarity, arrows or lines connecting the subtypes in the periphery to the central portion would be helpful.

Response: a) Thank you for this suggestion. Considering the nomenclature used in the endometrium research area, especially in single-cell studies, we would like to keep the widely used 'endometrial stromal cells' in this study. b) Number of RIF samples were added in the Methods section (line 715-717). c) Figure 1b was reorganized and additionally labeled to improve its readability.

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Thank you for addressing my concerns by performing additional analysis and validation.

Reviewer #3 (Remarks to the Author):

The authors have provided thorough and thoughtful responses to the Reviewers' comments and added some validation, which has added rigor to the data and its interpretation. Some additional items to enhance clinical translation of the findings as well as the issue of inferring causation vs association are noted below:

1. The additional patient metadata in Supplementary Table 1 and the response to Reviewer 3 about prior pregnancy history not impacting the results are appreciated. While the latter was a comment only to the Reviewer, adding this to the manuscript could be helpful to the reader, as this question may arise from both researchers of endometrial receptivity and clinicians who care for patients with infertility, RIF, and endometrial disorders.

2. In Methods, the endometrial biopsy was obtained with a Pipelle catheter with full suction from the fundus to the internal cervical os. Please explain if lower uterine segment or internal cervical tissue/cells were included in the samples. This is important as lower uterine segment endometrium differs from fundal endometrium in terms of steroid hormone response, and internal cervical os contains different cell types, either or both possibly contributing to heterogeneity among patient samples. Thus, comment is warranted if the sampling method included the lower uterine segment and internal cervical os (or not).

3. In the Methods, please state that E2 and P4 levels were not obtained in RIF donors. It is unclear.

4. Page, line 489 ff: as functional studies were not conducted, it is more appropriate to state that the observations are correlated with RIF, as opposed to "are causes of RIF". Please change here and other places in the manuscript where causation is invoked.

5. Line 597: While the authors state that the hyperinflammation of RIF endometrium might be a cause of abnormal progesterone signaling which was implicated in the regulation of endometrial inflammation, could it also be an effect of abnormal progesterone signaling?

6. Regarding the RIF endometrium stratification into two classes, displaced WOI and in-phase WOI with reduced epithelial receptivity and a hyper-inflammatory phenotype, more clarity is needed regarding the displaced WOI, as it could have implications clinically. Reviewer 1 had inquired about possible dysfunctional decidualization in a subset of RIF patients in class 1. The response was that RIF class 1 endometrium "was displaced to a later time interval with higher expression of decidualization and senescence genes". Please convey your insights into dysfunctionality in the displaced WOI, which seems like it is more than a shift of the "normal window". This could have clinical implications in that even adjusting for timing of ETs, as done with commercial ERA testing, not everyone gets pregnant. This could be due to other mechanisms underlying implantation failure – not solely the timing for the RIC Class I group (or a subgroup).

7. As the fertile controls all had conceptions in natural cycles except for IVF for genetic reasons, it is relevant to this manuscript to comment on what type of embryo transfer (ET) protocols were used for the IVF patients in the RIF group - were these fresh ETs or frozen ETs (FETs) with transfer in natural cycles (relevant to the findings of this paper), or "medicated cycles" (E2 and P4 with/without a GnRH analogue)? I raise this issue because results from this study could be relevant to timing of FETs in natural cycles (not medicated or other cycle preparations) and there is controversy about natural vs medicated FET cycles in terms of success rates and pregnancy/neonatal outcomes, cost, and convenience (PMID: 37172270).

Reviewer #3 (Remarks to the Author):

Response to Reviewer 1: comments warranting further clarification:

R1.9 Comment: *The information delivered here was to confirm that the non-immune subsets from different time points had highly overlapped predicted time while the stromal and unciliated epithelial cells had more separated predicted time.*

Does the author mean …. Confirm that the immune subsets…. As the stromal and unciliated epithelial cells are non-immune cells with subsets?

All other responses to R1 comments are appropriate.

Response to Reviewer 2: Comments warranting further response or clarification:

R2.2 comment: *Extensive and stringent digestion can impact drastically the transcriptomic signature too. In this case where authors chose a long digestion, it is particularly concerning since some of the key markers for cells populations are related to stress response-induced genes such as JUN/FOS like in stromal cells (Str3) or immune cells (NK, T cells in the StemVAE analysis). As well, DEG lists show the presence of mitochondrial genes and unannotated sequences or LncRNA within the top DEG. Can the author analyze the dissociation-induced stress response across different cell types/subtypes?*

The authors provide an appropriate response regarding the stress-response genes which are supported by other studies and importantly provide new IHC data of up-regulation of c-Jun expression in stromal cells in the implantation window, supporting the scRNA seq data are not a dissociation artifact.

However, the author did not address R2's comment about mitochondrial genes and unannotated sequence or LncRNA in the top DEGs. While the author's dissociation process is similar to what others have used in scRNA seq studies of the endometrium, comment on the mitochondrial genes and LnC RNAs and possible relationship in RIF vs controls would add rigor to the data interpretation but is not deemed essential.

R2.3 comment: *The analysis of the endometrium during the WOI is very interesting. However little information is provided around LH peak and sampling of the biopsies. Did the authors check LH levels at LH+1, +3,… to verify that all samples biopsies were collected at similar times related to the LH peak/individual? Were all LH peaks similar across patients?*

The authors don't answer the question directly. While they followed clinical markers of impending ovulation (ultrasound, estradiol), they had a single serum LH measurement around the time of the LH surge and chose levels of LH > 20 IU/mL to confirm the surge. The surge has an onset, a peak, and a decline within ~ 24 hours and defining it precisely is notoriously challenging, and levels of LH in the serum during the surge can vary by individual. Thus, the authors did not verify all patient biopsies were collected at precisely the same time but were approximately the same time. They should clarify that in the manuscript and that this could contribute to the large variability observed.

More important is that the authors admittedly did not measure serum estradiol and progesterone at the time of endometrial sampling on LH+3,+5,+7, +9, +11. This is an unfortunate and significant omission as it could have given insights into data interpretation and for readers' understanding in the context of circulating steroid hormone levels (a surrogate for endometrial levels which are rarely measured).

R2.4 Comment: *Cell type annotations EMT/MET: I find concerning that the authors claim the presence of EMT/MET cells without any further validation (IHC/IF at least should be used to confirm the existence of these cells in the tissue). How did the author discard the presence of doublets and potential mislabeling of these cells? Particularly, can the authors plot the EMT/MET cells back onto the main clustering? In the provided UMAP, a stromal (pink colored) subpopulation seem to cluster very closely from epithelial cells- is this the MET cluster?*

Additional data provided by the authors showed that ~25% of MET clustering to the major UMAP were in the stromal cell cluster close to the unciliated epithelial cells. Also, in the figure provided, the main stromal cluster shows a very high % of MET cells and the ciliated epithelial cells display a very high % of EMT, with unciliated les so. While some MET and EMT occurs in the endometrium, these %'s seem inflated and having another method of validation – e.g., pseudotime trajectory is important. The immunofluorescent staining is difficult to decipher in support of the high % of MET and EMT in the epithelial and stromal compartments, especially w/o control specimens and replicates and lack of clarity when in the cycle this figure was derived – implantation window, LH+7, LH+9, other?

R2.5 Comment: *The integration of spatial transcriptomics (Visium), albeit at a resolution different from single-cell analysis, was attempted using data from a single experiment and endometrial biopsy from a published manuscript, likely not corresponding to the time points analyzed in this study. I would suggest to validate cell populations, markers and CCC with a spatial transcriptomics/proteomic assay, single cell resolution if possible, using comparable samples and justifiable n.*

The authors indicate that they used a public endometrial ST data set to "help candidate the strong ligand-receptor pairs with spatial proximity in our own data". It is not clear to me how this was done and it is not explained in the response to R2.5. Moreover, the IHC figure is confusing, as SPPI, a receptivity marker, is said to be highly upregulated in LH+9 endometrium, but the figure shows barely visible immunostaining. Moreover, proximity of the CD-44+ CD45 immune cells to epithelial cells is demonstrated, but it is unclear how reproducible these data are, especially given the marked endometrial tissue variability in the samples used in the study as the authors show on page 12 of their rebuttal and the different cell types even in the same donor shown in table on pages 26 and 27 of the rebuttal.

R2.7 Comment: *Senescence: authors combined all cell subpopulations within a specific cell type to evaluate the putative senescence signature during the WOI - using SenMAyo, a senescence signature derived from human/mouse adipose tissue and bone marrow (not validated in teh endometrium). Does this apply to all stromal cell clusters? Surely there are key differences*

between stomal cell subpopulations, but do they all play a similar role in WOI and display similar senescence markers? Orthogonal validation would be good to confirm any of these claims.

The authors provide a reasonable reply to this comment. Of interest is whether the senescence scores in the WOI differ in different cell types in RIF vs controls and in RIF-class 1 vs class 2.

R2.9 Comment: *LGR5+ cells: authors suggest LGR5 is expressed in luminal/progenitor epithelial cells, but gene expression data shows no expression of LGR5 in the luminal compartment (Ext fig 2g). Additionally, the IF image (fig 1) suggest that LGR5 is also present in stromal cells, in addition to epithelial cells. Are those luminal or glandular cells? can you provide further details about RNA/protein detection of LGR5 within endometrial cells?*

In their rebuttal, the authors state that they did not detect many LGR5+ cells in the luminal compartment by scRNA seq, but they found high expression by IF in proliferative endometrium. It is unclear from the new immunofluorescent figure what cycle phase this is and how to interpret the IF staining in the luminal vs. glandular epithelium shown in the figure. Issue of # of replicates is key, along with control IF immunostaining.

R2.Comment 14: *While author conduct a thoughtful computational analysis of the immune/myeloid cells within RIF samples based on the obtained single cell data, they do not provide any functional validation/orthogonal validation to sustain the claims of correlation or hyperinflammation, nor the implication in impaired receptivity for these particular cell types.*

The authors' response to this comment is insufficient, as they commented that IFNG is highly expressed in immune cells and they found it highly upregulated (by qPCR) of *total* RNA of RIF vs. control samples. Moreover, they used a "receptive" epithelial cell line ISHIKAWA cells to model embryo attachment of trophoblast steroids that is inhibited by IFNG. Inferring that the upregulation in the immune cells is indirect and should be demonstrated, e.g., by IHC with appropriate immune cell markers. Note that the ISIKAWA cell line is an adenocarcinoma line, and reproducibility, # of independent experiments using the cell/spheroid model is unclear. Overall, I don't think that the authors have adequately addressed this comment from R2.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author)

Thank you for addressing my concerns by performing additional analysis and validation.

Response: Thank you again for the previous valuable comments which greatly improved the quality of the manuscript. And many thanks for the positive feedback this time.

Reviewer #3 (Remarks to the Author)

The authors have provided thorough and thoughtful responses to the Reviewers' comments and added some validation, which has added rigor to the data and its interpretation. Some additional items to enhance clinical translation of the findings as well as the issue of inferring causation vs association are noted below:

1. The additional patient metadata in Supplementary Table 1 and the response to Reviewer 3 about prior pregnancy history not impacting the results are appreciated. While the latter was a comment only to the Reviewer, adding this to the manuscript could be helpful to the reader, as this question may arise from both researchers of endometrial receptivity and clinicians who care for patients with infertility, RIF, and endometrial disorders.

Response: Thank you for this suggestion. We have added the comment about the prior pregnancy history not impacting the results into the Discussion section (line 512-516).

2. In Methods, the endometrial biopsy was obtained with a Pipelle catheter with full suction from the fundus to the internal cervical os. Please explain if lower uterine segment or internal cervical tissue/cells were included in the samples. This is important as lower uterine segment endometrium differs from fundal endometrium in terms of steroid hormone response, and internal cervical os contains different cell types, either or both possibly contributing to heterogeneity among patient samples. Thus, comment is warranted if the sampling method included the lower uterine segment and internal cervical os (or not).

Response: Thank you for the critical comment. First, we would like to clarify that the full suction for endometrial collection was performed when the Pipelle catheter was placed at the fundus under ultrasound guidance. The catheter was then moved downwards slowly during which negative pressure and spinning of the Pipelle catheter was applied to collect the endometrium. Since the negative pressure was applied at the fundus and most of the suction was done in the uterine cavity, the lower segment of the endometrium and

the cervical os was unlikely to contribute significantly in the collected samples. We have modified the description to avoid misunderstanding (line 666-667).

Second, our data showed clear dynamic expression patterns across the WOI for the stromal and epithelial cells, which to some extent validated that the poorly hormone-responsive low segment endometrium was not affecting our analyses. To demonstrate that cervical cells were not included in this dataset, we utilized a recently published endometrial dataset which identified a cervical epithelial cell cluster ($KRT5^+$) and a stromal cell cluster ($HOXA13^+$) [1]. Through cell mapping analysis, we found that only 3 out of 39,479 epithelial cells (1 from luminal, 2 from $EPCAM1^+SOX9^+$) and 30 out of 77,463 stromal cells (none from the small MET and proliferative clusters) in our dataset were predicted with the $KRT5^+$ epithelial identity and the $HOXA13^+$ stromal identity, respectively. The analysis shows that the cervical cell contamination is negligible.

3. In the Methods, please state that E2 and P4 levels were not obtained in RIF donors. It is unclear.

Response: For all the included donors (both fertile control and RIF donor), sample collection was conducted in a natural cycle. E2, P4 and LH levels were all assayed for determination of the surge day. Once the surge day was determined, hormonal levels were not measured for that cycle. We have modified the Methods to clarify this point (line 665, line 672-673).

4. Page, line 489 ff: as functional studies were not conducted, it is more appropriate to state that the observations are correlated with RIF, as opposed to “are causes of RIF”. Please change here and other places in the manuscript where causation is invoked.

Response: Thank you very much for the critical comment. We have changed the causation descriptions to more scientific correlation/association descriptions throughout the revised manuscript (line 493, line 611-613).

5. Line 597: While the authors state that the hyperinflammation of RIF endometrium might be a cause of abnormal progesterone signaling which was implicated in the regulation of endometrial inflammation, could it also be an effect of abnormal progesterone signaling?

Response: Thank you very much for this comment. We agree that the hyperinflammation of RIF might also be an effect of abnormal progesterone signaling as progesterone is an important regulator of

inflammatory response [2]. On the other hand, the abnormal inflammatory response can in turn regulate progesterone signaling [3]. Thus, we have modified the statement accordingly (line 611-613).

"Although the hyperinflammation of RIF endometrium might be a cause of abnormal progesterone signaling, their cause-and-effect relationship remains elusive since progesterone was implicated in the regulation of endometrial inflammation"

6. Regarding the RIF endometrium stratification into two classes, displaced WOI and in-phase WOI with reduced epithelial receptivity and a hyper-inflammatory phenotype, more clarity is needed regarding the displaced WOI, as it could have implications clinically. Reviewer 1 had inquired about possible dysfunctional decidualization in a subset of RIF patients in class 1. The response was that RIF class 1 endometrium "was displaced to a later time interval with higher expression of decidualization and senescence genes". Please convey your insights into dysfunctionality in the displaced WOI, which seems like it is more than a shift of the "normal window". This could have clinical implications in that even adjusting for timing of ETs, as done with commercial ERA testing, not everyone gets pregnant. This could be due to other mechanisms underlying implantation failure – not solely the timing for the RIC Class I group (or a subgroup).

Response: Thank you for the comment. In this manuscript, the displaced WOI represented those expression pattern resembled that of LH+9 though the samples were collected on LH+7. Based on the expression dynamics (Figure 3), the LH+9 stromal cells exhibited higher expression of decidualization and senescence genes. Thus, our response to Reviewer 1's comment is "*RIF class1 endometrium was displaced to a later time interval (mostly LH+9) with higher expression of decidualization and senescence genes*" when compared to LH+7. To determine whether the displaced WOI is more than a shift of the normal window, additional comparison with endometrium from the adjusted WOI is required. Currently, we only had data from a single time-point for the RIF samples which hinders further analysis. Our future pre-clinical testing of the method can clarify if other dysfunctionalities happen to the displaced WOI rather than a simple shift.

7. As the fertile controls all had conceptions in natural cycles except for IVF for genetic reasons, it is relevant to this manuscript to comment on what type of embryo transfer (ET) protocols were used for the IVF patients in the RIF group - were these fresh ETs or frozen ETs (FETs) with transfer in natural cycles (relevant to the findings of this paper), or "medicated cycles" (E2 and P4 with/without a GnRH analogue)? I raise this issue because results from this study could be relevant to timing of FETs in natural cycles (not

medicated or other cycle preparations) and there is controversy about natural vs medicated FET cycles in terms of success rates and pregnancy/neonatal outcomes, cost, and convenience (PMID: 37172270).

Response: Thank you so much for the critical comment. Although there were studies reporting comparable pregnancy rates for NC-FET and AC-FET, the conclusion remains controversial [4-7]. We totally agree that the embryo transfer protocols for RIF patients is important. In real world, it is hard to include RIF patients only with identical history of endometrium preparation for embryo transfer. In our study, the endometrium preparation protocol for RIF donors was heterogenous including 'Fresh embryo transfer', "Stimulated natural cycle" "HRT cycle", and "GnRHa+HRT cycle" (details shown below), and each patient had at least 1 FET with natural cycle. We collected endometrium from a natural cycle of all the donors and aimed at investigating the natural state of the RIF endometrium. Thus, the indication of this study should be for natural cycles rather than medicated cycles despite the heterogenous protocols used. Future single-cell transcriptomic comparison between endometria from natural cycle and HRT cycle is warranted.

Original Donor ID	Sample ID	Times of implantation failure	Embryos with PGT	Transfer cycles
E01	RIF_1	4	1	2 NC-FET, 1 Stimulated-FET, 1 HRT-FET
E07	RIF_2	4	4	4 NC-FET
E18	RIF_3	3	0	2 NC-FET, 1 HRT-FET
E28	RIF_4	3	1	1 HRT-FET, 2 NC-FET
E30	RIF_5	5	1	1 NC-FET, 1 Stimulated-FET, 1 HRT-FET, 2 GnRHa-HRT-FET
E02	RIF_6	3	0	1 fresh ET, 1 NC-FET, 1 HRT-FET
E05	RIF_7	4	1	2 NC-FET, 1 Stimulated-FET, 1 HRT-FET
E31	RIF_8	4	1	2 NC-FET, 1 HRT-FET, 1 GnRHa-HRT-FET
E32	RIF_9	3	2	3 NC-FET
E33	RIF_10	3	2	1 NC-FET, 2 HRT-FET

Response to Reviewer 1: comments warranting further clarification:

R1.9 Comment: The information delivered here was to confirm that the non-immune subsets from different time points had highly overlapped predicted time while the stromal and unciliated epithelial cells had more separated predicted time.

Further comment by Review3 on our 1st response: "*Does the author mean .... Confirm that the immune subsets.... As the stromal and unciliated epithelial cells are non-immune cells with subsets?*

All other responses to R1 comments are appropriate."

Response: Thank for pointing out this point. It was a typo. We mean to confirm that the immune subsets rather than non-immune subsets had highly overlapped predicted time.

Response to Reviewer 2: Comments warranting further response or clarification:

R2.2 comment: Extensive and stringent digestion can impact drastically the transcriptomic signature too. In this case where authors chose a long digestion, it is particularly concerning since some of the key markers for cells populations are related to stress response- induced genes such as JUN/FOS like in stromal cells (Str3) or immune cells (NK, T cells in the StemVAE analysis). As well, DEG lists show the presence of mitochondrial genes and unannotated sequences or LncRNA within the top DEG. Can the author analyze the dissociation- induced stress response across different cell types/subtypes?

Further comment by Review3 on our 1st response: "*The authors provide an appropriate response regarding the stress-response genes which are supported by other studies and importantly provide new IHC data of up-regulation of c-Jun expression in stromal cells in the implantation window, supporting the scRNA seq data are not a dissociation artifact.*

However, the author did not address R2's comment about mitochondrial genes and unannotated sequence or LncRNA in the top DEGs. While the author's dissociation process is similar to what others have used in scRNA seq studies of the endometrium, comment on the mitochondrial genes and LnC RNAs and possible relationship in RIF vs controls would add rigor to the data interpretation but is not deemed essential.

Response: Thank you for the further comment. As the mitochondrial genes are related to metabolic stress under an inflammation status [8], and the lncRNA NEAT1 has various reported functions including regulating stress and inflammatory response [9,10], they together with other top DEGs may indicate a link between inflammation and epithelial receptivity in RIF. We have added discussion on this point in the revised manuscript (line 609-611).

"Moreover, the top dysregulated genes (Extended Data Fig. 10e) found in RIF involving stress and immune response indicated the link between hyper-inflammation and reduced epithelial receptivity."

R2.3 comment: The analysis of the endometrium during the WOI is very interesting. However little information is provided around LH peak and sampling of the biopsies. Did the authors check LH levels at LH+1, +3,... to verify that all samples biopsies were collected at similar times related to the LH peak/individual? Were all LH peaks similar across patients?

Further comment by Review3 on our 1st response: The authors don't answer the question directly. While they followed clinical markers of impending ovulation (ultrasound, estradiol), they had a single serum LH measurement around the time of the LH surge and chose levels of LH > 20 IU/mL to confirm the surge. The surge has an onset, a peak, and a decline within ~ 24 hours and defining it precisely is notoriously challenging, and levels of LH in the serum during the surge can vary by individual. Thus, the authors did not verify all patient biopsies were collected at precisely the same time but were approximately the same time. They should clarify that in the manuscript and that this could contribute to the large variability observed.

More important is that the authors admittedly did not measure serum estradiol and progesterone at the time of endometrial sampling on LH+3,+5,+7, +9, +11. This is an unfortunate and significant omission as it could have given insights into data interpretation and for readers' understanding in the context of circulating steroid hormone levels (a surrogate for endometrial levels which are rarely measured).

Response: Thank you so much for the critical comment. We must have conveyed unclear information in our response to R2.3. We would like to clarify that daily LH levels together with P4 and E2 around the ovulation day were measured to determine the LH surge (as described in the Method, line 669-674). We agreed that the biopsies were collected at similar rather than the exact same time. Moreover, we can see from our study and others that the LH surge levels, and peak patterns varied a lot among patients. Thus, using LH level to verify that all the biopsies were collected at the same times relative to the LH peak in each donor is challenging or even not possible. Therefore, as suggested, we further discussed this point in the revised manuscript (line 516-518).

"While the pregnancy history effect was to some extent ruled out, it remains possible that the cellular heterogeneity might be due to the similar rather than precise sampling timing which is notoriously challenging."

With respective to the missing of hormone determination at biopsy, we have discussed the necessity to obtain the hormone levels at each biopsy for endometrium research in the 1st revision (line 596-598, yellow highlighted).

R2.4 Comment: Cell type annotations EMT/MET: I find concerning that the authors claim the presence of EMT/MET cells without any further validation (IHC/IF at least should be used to confirm the existence of these cells in the tissue). How did the author discard the presence of doublets and potential mislabeling of these cells? Particularly, can the authors plot the EMT/MET cells back onto the main clustering? In the provided UMAP, a stromal (pink colored) subpopulation seem to cluster very closely from epithelial cells- is this the MET cluster?

Further comment by Review3 on our 1st response: Additional data provided by the authors showed that ~25% of MET clustering to the major UMAP were in the stromal cell cluster close to the unciliated epithelial cells. Also, in the figure provided, the main stromal cluster shows a very high % of MET cells and the ciliated epithelial cells display a very high % of EMT, with unciliated less so. While some MET and EMT occurs in the endometrium, these %'s seem inflated and having another method of validation – e.g., pseudotime trajectory is important. The immunofluorescent staining is difficult to decipher in support of the high % of MET and EMT in the epithelial and stromal compartments, especially w/o control specimens and replicates and lack of clarity when in the cycle this figure was derived – implantation window, LH+7, LH+9, other?

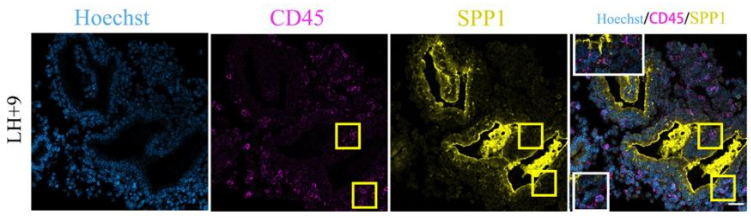
Response: Thank you for the comment. First, we would like to clarify that although 25% of the whole MET cluster located closely to the epithelial cells in the main UMAP, 93% of these 25% MET cluster in the latent space were from the whole stromal cell population. This is to answer the reviewer 2's question about the MET identity of the pink colored stromal subpopulation located nearby the epithelial cells. Second, the inflated proportion reflected by the highlighted UMAP is caused by the enlarged dot size of the highlighted cells. The proportion of MET in stromal cells and EMT in epithelial cells based on our scRNA-seq data is NOT high at 2.03%, and 2.13%, respectively. As shown in Figure 1d, the EMT cluster exhibited high differentiation potentials. For the immunostaining result, only the positive results were shown. We performed experiment in a total of 7 samples (1x LH+3, 1x LH+5, 3x LH+7, 1x LH+9, 1x LH+11). Among these samples, cells co-expressing epithelial cell marker and stromal cell marker were found in 1 LH+5, 1 LH+7 and 1 LH+9 samples. As we cannot definitively conclude the absence of EMT/MET in the other samples based on the negative staining result because only part of the whole uterus was checked, we think that it might be better to only show the positive presence of potential EMT/MET in the figure. We have added detailed experimental information in the 2nd revised manuscript (Figure 1f legend).

R2.5 Comment: The integration of spatial transcriptomics (Visium), albeit at a resolution different from single-cell analysis, was attempted using data from a single experiment and endometrial biopsy from a published manuscript, likely not corresponding to the time points analyzed in this study. I would suggest to validate cell populations, markers and CCC with a spatial transcriptomics/proteomic assay, single cell resolution if possible, using comparable samples and justifiable n.

Further comment by Review3 on our 1st response: The authors indicate that they used a public endometrial ST data set to "help candidate the strong ligand-receptor pairs with spatial proximity in our own data". It is not clear to me how this was done and it is not explained in the response to R2.5. Moreover, the IHC figure is confusing, as SPPI, a receptivity marker, is said to be highly upregulated in LH+9 endometrium, but the figure shows barely visible immunostaining. Moreover, proximity of the CD-44+ CD45 immune cells to epithelial cells is demonstrated, but it is unclear how reproducible these data are, especially given the marked endometrial tissue variability in the samples used in the study as the authors show on page 12 of their rebuttal and the different cell types even in the same donor shown in table on pages 26 and 27 of the rebuttal.

Response: Thank you for the comment. We firstly identified significant ligand-receptor interactions (initial LRI set) in the single-cell transcriptomic data using the R package CellChat (v.1.6.1). Simultaneously, spatially co-expressed ligand-receptor pairs (spatial LRI set) for endometrium were identified with the Python package SpatialDM (v.0.1.0) utilizing a public spatiatranscripomic data of secretory endometrium deposited in the ArrayExpress (accession number E-MTAB-9260). The initial LRI set was further candidate if they were also present in the spatial LRI set.

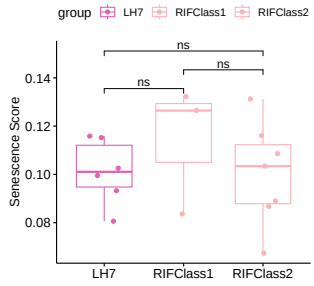
For the IHC figure, SPP1 (red) was highly expressed in the glands ($EPCAM^+$ cells in green) at LH+9 but not at LH+3. We wonder whether it is the contrast problem. Here we showed another figure (below) which demonstrated the high SPP1 expression at LH+9. The proximity staining was only performed in two independent samples because of the limited number of sections available and the results were reproducible. The number of samples used in this experiment has been added in the revised manuscript (Supplementary Fig. 6).



R2.7 Comment: Senescence: authors combined all cell subpopulations within a specific cell type to evaluate the putative senescence signature during the WOI - using SenMAyo, a senescence signature derived from human/mouse adipose tissue and bone marrow (not validated in the endometrium). Does this apply to all stromal cell clusters? Surely there are key differences between stomal cell subpopulations, but do they all play a similar role in WOI and display similar senescence markers? Orthogonal validation would be good to confirm any of these claims.

Further comment by Review3 on our 1st response: The authors provide a reasonable reply to this comment. Of interest is whether the senescence scores in the WOI differ in different cell types in RIF vs controls and in RIF-class 1 vs class 2.

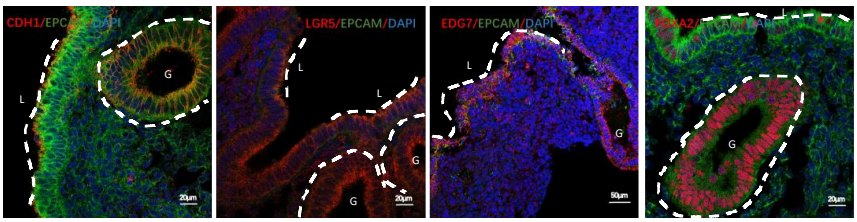
Response: Thank you for this comment. We performed the senescence score comparison for stromal cells among different groups and it did not show statistically significant difference (Extended figure 10b). This was also discussed in the original manuscript (lines 602-606, yellow highlighted).

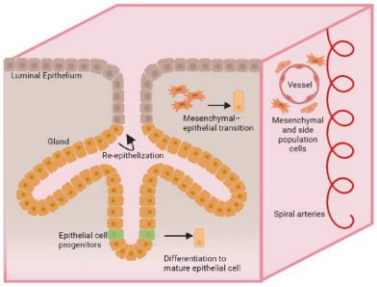


R2.9 Comment: LGR5+ cells: authors suggest LGR5 is expressed in luminal/progenitor epithelial cells, but gene expression data shows no expression of LGR5 in the luminal compartment (Ext fig 2g). Additionally, the IF image (fig 1) suggest that LGR5 is also present in stromal cells, in addition to epithelial cells. Are those luminal or glandular cells? can you provide further details about RNA/protein detection of LGR5 within endometrial cells?

Further comment by Review3 on our 1st response: In their rebuttal, the authors state that they did not detect many LGR5+ cells in the luminal compartment by scRNA seq, but they found high expression by IF in proliferative endometrium. It is unclear from the new immunofluorescent figure what cycle phase this is and how to interpret the IF staining in the luminal vs. glandular epithelium shown in the figure. Issue of # of replicates is key, along with control IF immunostaining.

Response: Thank you for this comment. Sorry for our missing information in the responses to R2.9. The new figure was based on secretory phase samples complimentary to that from proliferative phase samples in Figure 1 as requested by Reviewer 2. To make it clear, labels were added to the new immunofluorescence figure which was based on secretory phase samples (Supplementary Fig. 1, also shown below, G: gland, L: lumen). Here, one ambiguous point is that the potential gland formed by the extension of luminal epithelial cells (the second and third panel) is hard to determine as they contain both luminal and glandular cells in it (a schematic figure was shown below). Therefore, they were only partially labeled. For each phase, 3 samples were included. For the immunostaining, we always included technical negative controls (secondary antibody alone). The number of samples used have been added in the revised manuscript (Supplementary Fig. 1).





(this schematic figure is from *Tabeeva, et al.* [11])

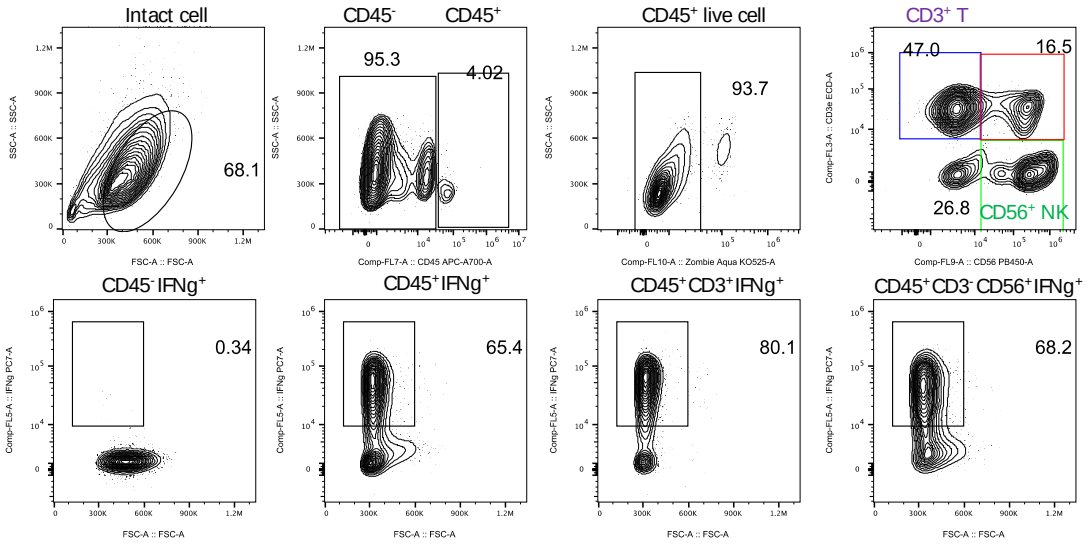
R2.Comment 14: While author conduct a thoughtful computational analysis of the immune/myeloid cells within RIF samples based on the obtained single cell data, they do not provide any functional validation/orthogonal validation to sustain the claims of correlation or hyperinflammation, nor the implication in impaired receptivity for these particular cell types.

Further comment by Review3 on our 1st response: The authors' response to this comment is insufficient, as they commented that IFNG is highly expressed in immune cells and they found it highly upregulated (by qPCR) of total RNA of RIF vs. control samples. Moreover, they used a "receptive" epithelial cell line ISHIKAWA cells to model embryo attachment of trophoblast steroids that is inhibited by IFNG. Inferring that the upregulation in the immune cells is indirect and should be demonstrated, e.g., by IHC with appropriate immune cell markers. Note that the ISIKAWA cell line is an adenocarcinoma line, and

reproducibility, # of independent experiments using the cell/spheroid model is unclear. Overall, I don't think that the authors have adequately addressed this comment from R2.

Response: Thank you so much for this critical comment. The difficulties in detecting IFNG protein using antibody-based method including flow-cytometry have been documented [12]. Despite of this, we still had tested the IFNG antibody (Proteintech, 15365-1-AP) with different conditions for immunofluorescent detection of IFNG protein in the paraffin sections of human endometrium but without success (no signal). Meanwhile, we also tested the antibody (BD Pharmingen™ PE-Cy™7 Mouse Anti-Human IFN-γ) for flow cytometry in human endometrium. We had tried our best to newly collect 3 fresh samples of mid-secretory stage (shown in the table below). Although these samples couldn't represent the healthy endometrium as well as the recurrent implantation failure, they still helped us to have the overall sense of IFNG expression. The results showed positive IFN-γ detection in endometrial immune cells but negligible detection in the non-immune subset (below), consistent with what we found in the single-cell transcriptomic datasets (Supplementary Fig 10c) as well as with previous publications [13-15]. Therefore, utilizing the cell remains from single-cell suspensions which showed high expression of IFNG transcript in the total RNA of RIF group by qPCR (Figure 6e) to some extent supported the data analysis result. But we admitted the insufficiency and discussed this limitation in the revised manuscript (line 628-631, as below).

"Although the increased transcriptomic and protein expression of Interferon gamma was previously detected in RIF endometrium and peripheral blood, respectively, direct IFNG protein detection in RIF endometrial immune cells should be warranted in the future."



Donor	Pregnancy history	$CD45^-$	$CD45^+$	$CD45^+CD3^+CD56^-$	$CD45^+CD3^-CD56^+$
PMZ	3 pregnancy losses	0.34	65.4	80.1	68.2
NJM	3 pregnancy losses	0.17	74.6	84	83
YTT	2 pregnancy losses	0.31	44.7	73.4	65.5

ISHIKAWA cell line is considered as receptive epithelium and has been widely used for the *in vitro* study of human implantation [16-18]. Therefore, we also chose it to conduct the *in vitro* attachment experiment treated with IFNG proteins. The experiments were independently replicated in three times (each time, 2 technical replicates were included) which has been added to this revised manuscript (Supplementary Fig. 10).

As further study of the role of IFNG in endometrium requires large sample size because of the large variation, we sincerely admitted that recruitment of new and enough eligible samples within a short duration is not feasible. But we have our interests in the following study of IFNG in the human endometrium. Therefore, future long-term functional and mechanistic study with new samples and models are warranted.

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3 Zhang, P. & Wang, G. Progesterone Resistance in Endometriosis: Current Evidence and Putative Mechanisms. *Int J Mol Sci* **24**, doi:10.3390/ijms24086992 (2023).

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11 Tabeeva, G. *et al.* The Therapeutic Potential of Multipotent Mesenchymal Stromal Cell-Derived Extracellular Vesicles in Endometrial Regeneration. *Int J Mol Sci* **24**, doi:10.3390/ijms24119431 (2023).
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16 Yue, C. *et al.* Human embryonic stem cell-derived blastocyst-like spheroids resemble human trophectoderm during early implantation process. *Fertil Steril* **114**, 653-664 e656, doi:10.1016/j.fertnstert.2020.01.009 (2020).
17 Vergaro, P., Tiscornia, G., Rodriguez, A., Santalo, J. & Vassena, R. Transcriptomic analysis of the interaction of choriocarcinoma spheroids with receptive vs. non-receptive endometrial epithelium cell lines: an in vitro model for human implantation. *J Assist Reprod Genet* **36**, 857-873, doi:10.1007/s10815-019-01442-9 (2019).
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REVIEWER COMMENTS

Reviewer #3 (Remarks to the Author):

The authors have largely responded to comments by R3. However, there is one item that needs attention:

Reviewer 3 comment on response to R2.4 Comment

This is the authors' rebuttal to this comment:

a) "As shown in Figure 1d, the EMT cluster exhibited high differentiation potentials. For the immunostaining result, only the positive results were shown. We performed experiment in a total of 7 samples (1x LH+3, 1x LH+5, 3x LH+7, 1x LH+9, 1x LH+11). Among these samples, cells co-expressing epithelial cell marker and stromal cell marker were found in 1 LH+5, 1 LH+7 and 1 LH+9 samples. As we cannot definitively conclude the absence of EMT/MET in the other samples based on the negative staining result because only part of the whole uterus was checked, we think that it might be better to only show the positive presence of potential EMT/MET in the figure.

b) We have added detailed experimental information in the 2nd revised manuscript (Figure 1f legend)."

My comments:

a) If the results were equivocal, showing only the positive results can be misleading, and the authors should either delete the positive result or state that there was variability of positive and negative staining and thus the results are preliminary and need to be validated in more extensive studies.

b) This reviewer doesn't see anything new in Fig 1f legend. Including a statement about variability of the results would be appropriate here.

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Response: Thank you so much for this critical comment. As suggested, we chose to point out the patient variability which should be very important to the readers. We modified the statement in the main text (line 178-189) as well as in the figure legend (line 1164-1165):

Main text:

"Potential EMT/MET cells co-expressing epithelial cell marker EPCAM and stromal cell marker VIM were also found through immunostaining (Fig. 1f) but with patient variability which required future extensive study."

Figure Legend

"Immunostaining of epithelial cell marker EPCAM and stromal cell marker VIM in a LH+7 endometrial tissue (Patient variability existed regarding EPCAM-VIM co-expressing cells, only positive staining shown here)."

REVIEWERS' COMMENTS

Reviewer #3 (Remarks to the Author):

The author has appropriately commented that the data in question shown in Figure 1f are variably expressed among samples and stated that more study in the future is warranted.