nature portfolio

Peer Review File

Mesenchymal-to-epithelial transition of perivascular cells contributes to endometrial re-epithelialization

Corresponding Author: Dr Tony DeFalco

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
SUMMARY

This manuscript utilizes a genetic lineage tracing model to investigate novel mechanisms of epithelial remodeling and repair in the mouse uterus. The results support the idea that Nestin+ perivascular cells can be activated under certain conditions to differentiate into epithelial cells.

OVERALL AND MAJOR COMMENTS

This manuscript contains a series of studies that address the role of a new perivascular mesenchymal cell population in endometrial epithelium remodeling and regeneration. For the most part, the studies are appropriate in terms of design and analyses as well as interpretation of the results. A major issue is the quality of the figures in that some of the photomicrographs are blurry and not in focus. The results provide some new information on endometrial regeneration, repair and homeostasis.

Major Comments:
1) The Introduction is overly long and could be shortened with focus.
2) Tamoxifen is a mixed estrogen receptor agonist and antagonist depending on the tissue. Is the dosing regimen to activate the genetic labeling approach problematic given this knowledge? Could there be lingering effects of the tamoxifen that are somewhat addressed in the Discussion?
3) The quality of many of the photomicrographs is problematic given they are not in focus. The blurriness and lack of focus is most problematic in those with higher magnification.
4) Figure 2: Were the uteri collected at the same stage of the estrous cycle?
5) Have the authors tried a wounding assay in the uterus, such as scratching the uterine lumen with a burred needle, to investigate the contributions of Nestin+ perivascular cells in reepithelialization of the endometrium lining?
6) Figure 4: E2 administration to an ovariectomized and rested mouse elicits epithelial proliferation, whereas P4 does not. Thus, the result is not entirely surprising.

SPECIFIC COMMENTS

Line Comment
129 Is it true that significant turnover occurs in the endometrial epithelium during the estrous cycle (Reference 39)?

139 “Subset” should be changed to “some”

202 Artificial decidualization elicits the formation of an artificial deciduoma rather than decidua. Thus, it may be more appropriate to use the term deciduoma tissue rather than decidua or decidualized tissues. There are gene expression differences between a true decidua elicited by embryo implantation as compared to a deciduoma formed by intrauterine oil injection, intrauterine scratch, or uterine horn crush.

231 Use the accepted gene nomenclature for estrogen receptor alpha that is ESR1 rather than ER alpha

225 If a lower number of Tomato+ cells were seeded into the Matrigel, then one would expect lower organoid formation efficiency. Stromal and mesenchymal cells will not survive in Matrigel and the medium used for EMO expansion, thus is the observed outcome simply due to lower numbers of Tomato+ cells being seeded as compared to the control?

273 Which ERs? ESR1 or ESR2 or both?

Figure S1B and S1F The CDH1 IF photomicrograph appears out of focus, particularly the higher magnification in S1F of the epithelium.

Figure S2 Some of the photomicrographs appear out of focus and blurry.

Figure S3A This photomicrograph is of the implantation site, but what happens in the interimplantation sites where the epithelium remains intact and does not have to be regenerated?

Figure S4D and S4E The vimentin (VIM) IF is not convincing and appears as background or autofluorescence. The quality of the photomicrographs in S4D is poor and not in focus.

Figure S4F The ESR1 IF is not convincing. Please show the ESR1 IF channel only independent of DAPI.

Reviewer #2

(Remarks to the Author)
The origin of the endometrial epithelium has long been a fundamental question in female reproductive biology. Previous studies suggest that epithelial regeneration arises from multiple distinct sources. This study uncovers an unexpected role of Nestin+ perivascular cells in epithelial regeneration via mesenchymal-to-epithelial transition (MET), using genetic lineage tracing assays in mice. The authors demonstrate that Nestin+ perivascular cells function as progenitor cells, differentiating into epithelial cells to restore the uterine lining after epithelial shedding during the estrous cycle, pregnancy, and artificial decidualization—a model mimicking menstruation.

Mechanistically, this study further explores the molecular pathways regulating this process. The findings reveal that Notch signaling maintains Nestin+ cells in a quiescent state, while estrogen signaling, mediated by estrogen receptor alpha (ERα), suppresses Notch activity to activate these cells and drive their differentiation. Through immunofluorescence imaging, flow cytometry, and quantitative PCR analyses, the study identifies a crucial Estrogen-Notch signaling axis that governs the transition of perivascular cells into epithelial cells.

These findings have significant implications for reproductive health, as impaired endometrial regeneration is associated with conditions such as infertility, endometriosis, and endometrial hyperplasia. By identifying perivascular cells as a novel epithelial progenitor population, this study lays the groundwork for potential therapeutic strategies aimed at enhancing uterine regeneration.

Minor Comments:
Since Axin2-positive cells have been established as another key source of epithelial regeneration, please provide a more detailed comparison of these two progenitor populations in the discussion section.

The authors speculate that ERα inhibits the expression of Notch-associated genes. Given that genome-wide ERα distribution data in the uterus is available, is there direct evidence of ERα binding to these genes?

If possible, move Figure S7 to the main figure panel to enhance reader comprehension of the study's findings.

Label Figures 3D, 7A and S2B to clearly distinguish the two rectangles.

Reviewer #3

(Remarks to the Author)
The manuscript by Li et al. presents evidence that Nestin-positive perivascular cells participate in endometrial epithelial homeostasis and regeneration through mesenchymal-to-epithelial transition. By using mouse genetic models and both in vitro and in vivo lineage-tracing approaches, they convincingly demonstrate an additional epithelial progenitor cell source beyond previously characterized basal epithelial and stromal populations. These insights advance our understanding of re-epithelialization in the endometrium and underscore how perivascular cell plasticity may contribute to tissue repair following natural or induced endometrial shedding. Although this is a scientifically sound manuscript, I have several concerns, that if addressed, will strengthen the manuscript
1. While the authors explore a role for estrogen in activating perivascular cells, studies in rodents and primates have demonstrated that endometrial repair occurs independently of sex hormones (Hartman 1944; Tu'uhevaha J Kaitu'u-Lino , et al. 2007; Kaitu'u-Lino et al. 2010). The authors should acknowledge these findings and more directly discuss how hormone dispensability or context-specific hormone requirements might intersect with their proposed mechanism of Nestin-positive perivascular cell–driven re-epithelialization.
2. Given that a core aspect of this study hinges on microscopic evaluation of lineage-traced cells, higher-resolution, more clearly labeled images would provide stronger evidence. This issue may have been partially impact due to image size restrictions at submisiion
3. In line 170, the authors state, "Interestingly, we also observed a Tomato+ cell half-embedded in the epithelial layer, suggesting it was undergoing a transition." It is unclear whether this is a routine observation or if it is a rare observatiion. Providing quantitative data (would be valuable. In addition, clarifying whether these cells show any known MET markers would solidify the claim that they are actively transitioning to an epithelial phenotype.
4. The authors mainly rely on immunofluorescence to assess the presence and number of Nestin-derived cells, particularly where the overall numbers appear low. While this is acceptable in some instances, flow cytometry would offer a more robust and quantitative measure of these rare populations.
5. The methods for organoid establishment need to be expanded, particularly regarding cell seeding density. Differing numbers of plated cells across experimental conditions could confound comparisons of organoid-forming efficiency.
6. The presence of tdTomato-positive cells in "control" organoids needs further clarification. It would be helpful to confirm whether any tamoxifen exposure or lineage-leakage effects led to unexpected labeling, and to elaborate on how promoter strength variations might explain delayed or minimal epithelial labeling. In addition, negative selection of CDH1 or EPCAM before plating tdTomato-positive perivascular cells would provide a cleaner starting population, allowing stronger conclusions about perivascular to epithelial transition in vitro.
7. The authors should address potential confounding influences of tamoxifen treatment itself on Nestin expression or on the uterus more broadly. Although the authors have taken precautions to mitigate tamoxifen's antiestrogenic effects, validating that Nestin expression in the uterine epithelium is not an artifact of tamoxifen treatment is essential. This could be done by qRT-PCR or ISH.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
SUMMARY

This manuscript utilizes a genetic lineage tracing model to investigate novel mechanisms of epithelial remodeling and repair in the mouse uterus. The results support the idea that Nestin+ perivascular cells can be activated under certain conditions to differentiate into epithelial cells.

OVERALL AND MAJOR COMMENTS

The revised manuscript is improved and has addressed many of the major and minor comments based on review of the initial manuscript.

Major Comments:
1) Endometrial regeneration after parturition occurs in the absence of estrogen, thus estrogen is not always the driver of endometrial regeneration.
2) Figures (Main and Supplementary): The major cell types in one or more panels of the figures need to be clearly labeled in the sections to enhance interpretation and readability. For instance, LE, GE, Stroma, etc. This is correctly done in Supplementary Figure 2.
3) Figures: Please indicate in figure legend or text the type of section used for immunolocalization such as transverse or longitudinal.
4) Figure 5: The higher magnification photomicrographs in Panel a (middle and right) are not in focus and blurry.

SPECIFIC COMMENTS

Line Comment
22 The epithelial cells undergo apoptosis during the estrous cycle…they are not shed
37 see above comment

78 Please use only the accepted gene nomenclature of ESR1
108 stained should be immunostained
138 “antimesometrial stroma” not stroma of the anti-mesometrium
364 Add “potentially” before contribute
453 Avoid using theatrical terms such as “drastic” and “dramatic”

Reviewer #2

(Remarks to the Author)
The response addresses all of my concerns—no further questions.

Reviewer #3

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

Version 2:

Reviewer comments:

Reviewer #1

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

Reproductive Sciences Center
Division of Developmental Biology

Tony DeFalco, Ph.D.
Associate Professor
Co-Director

Location S, Room 216
3333 Burnet Avenue, MLC 7045
Cincinnati, OH 45229-3039
Phone: (513) 803-3988
Fax: (513) 803-1160
Email: Tony.DeFalco@cchmc.org

June 19, 2025

RE: Manuscript ID #NCOMMS-25-10067-T

Dear Reviewers,

Reviewer comments are in black font and our responses are listed below in blue font.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

SUMMARY

This manuscript utilizes a genetic lineage tracing model to investigate novel mechanisms of epithelial remodeling and repair in the mouse uterus. The results support the idea that Nestin+ perivascular cells can be activated under certain conditions to differentiate into epithelial cells.

OVERALL AND MAJOR COMMENTS

This manuscript contains a series of studies that address the role of a new perivascular mesenchymal cell population in endometrial epithelium remodeling and regeneration. For the most part, the studies are appropriate in terms of design and analyses as well as interpretation of the results. A major issue is the quality of the figures in that some of the photomicrographs are blurry and not in focus. The results provide some new information on endometrial regeneration, repair and homeostasis.

Thank you for acknowledging the novelty and scientific merit of our findings regarding perivascular mesenchymal cells in endometrial regeneration. We agree that clarity of the images is critical for their accurate interpretation. We have carefully reviewed all figures and have replaced blurry photomicrographs with higher-resolution images.

Major Comments:
1) The Introduction is overly long and could be shortened with focus.

Thank you for the suggestion. We have shortened and revised the Introduction to improve its clarity and focus.

2) Tamoxifen is a mixed estrogen receptor agonist and antagonist depending on the tissue. Is the dosing regimen to activate the genetic labeling approach problematic given this knowledge? Could there be lingering effects of the tamoxifen that are somewhat addressed in the Discussion?

Tamoxifen can have tissue-specific estrogenic or anti-estrogenic effects. In the uterus, tamoxifen can act as an estrogen agonist, meaning it can stimulate the growth of uterine tissue (PMID: 32906618). In our study, we used a dosing regimen of 75 mg/kg, which is the standard concentration recommended by The Jackson Laboratory for effective CreER activation. We initially tested a single injection of 4-hydroxytamoxifen (4-OHT) but observed very low recombination efficiency (Supplementary Fig. 1d). While three consecutive daily 4-OHT injections increased efficiency, they also caused adverse effects such as abdominal fat adhesions. The current two-injection protocol was an optimal balance to achieve efficient CreER-mediated recombination while minimizing physiological impacts. We have discussed the dosing rationale in the Discussion section on lines 393-400, including statements such as: "We attempted to enhance this efficiency by further increasing either the 4-OHT dose or the number of exposure days to 4-OHT. However, these regimens led to undesirable side effects: peritoneal adhesions, infertility, and failure to induce decidualization due to tamoxifen's antiestrogenic effect in perturbing pregnancy." Furthermore, in the Discussion we also noted that, for postpartum assays, 4-OHT treatment took place far in advance (2 months) of mating, to minimize any potentially detrimental effects (lines 397-400).

3) The quality of many of the photomicrographs is problematic given they are not in focus. The blurriness and lack of focus is most problematic in those with higher magnification.

We agree with the reviewer, and we have ensured that high-resolution photomicrographs are provided. However, we noticed that figure resolution decreased once the PDF was compiled for reviewers during the initial submission, which potentially made figures appear blurry. We have provided individual high-resolution figures for the revised version of the manuscript. We will work with the editors to maintain the figure resolution if the manuscript is accepted.

4) Figure 2: Were the uteri collected at the same stage of the estrous cycle?

Uteri were exposed to 4-OHT during diestrus, except for samples shown in Fig. 2j and 2k (estrus phase). The uteri were collected based on days post-injection without controlling for estrous cycles. We reason that if MET took place, Tomato^{+} epithelial cells should be detected regardless of the stage of the estrous cycle.

5) Have the authors tried a wounding assay in the uterus, such as scratching the uterine lumen with a burred needle, to investigate the contributions of Nestin+ perivascular cells in reepithelialization of the endometrium lining?

We appreciate this suggestion. However, we have not been able to establish a scratching protocol which generates consistent wounding. We are concerned that variation in the extent of scratches would compromise the rigor and reproducibility of our results and that any results would be inconclusive. As well, it is likely that distinct mechanisms are used for wounding repair versus what is seen during estrous cycles and postpartum. Therefore, we have not performed a mechanical wounding assay in the current study, but we agree it would be a valuable direction for future investigation.

6) Figure 4: E2 administration to an ovariectomized and rested mouse elicits epithelial proliferation, whereas P4 does not. Thus, the result is not entirely surprising.

We agree with the reviewer that E2 stimulates epithelial proliferation. However, Tomato$^+$ and Tomato-negative epithelial cells should all proliferate in response to E2 treatment. If they respond to E2 similarly, the percentage of Tomato$^+$ cells should remain similar even though the total epithelial cell number is increased after E2 treatment. However, we observed the percentage of Tomato$^+$ epithelial cells is increased, suggesting relatively more Tomato$^+$ perivascular cells are incorporated into the epithelial layer. A formally possible alternative explanation is that Tomato$^+$ epithelial cells are more sensitive to estrogen stimulation, although that scenario is not likely to be the case. We did not make our conclusion clear in our description of the results in Fig. 4, so we revised our description of the results (lines 231-244). Ultimately, this experiment simply aimed to confirm E2's role in inducing the differentiation of Nestin$^+$ perivascular cells into epithelial cells.

SPECIFIC COMMENTS

Line Comment

129 Is it true that significant turnover occurs in the endometrial epithelium during the estrous cycle (Reference 39)?

Yes, significant turnover does occur in the endometrial epithelium during the estrous cycle. As the reviewer mentioned in comment #6 above, estrogen elicits epithelial proliferation. In proestrus and estrus, epithelial cells undergo rapid cell proliferation. And by diestrus, the uterine luminal epithelium shrinks back to a slit-like opening. We believe these changes in the uterine epithelial structure throughout the estrous cycle require a major fluctuation in epithelial cell number and, thus, significant turnover.

139 "Subset" should be changed to "some"

As requested, we have changed "subset" to "some" (line 123).

202 Artificial decidualization elicits the formation of an artificial deciduoma rather than decidua. Thus, it may be more appropriate to use the term deciduoma tissue rather than decidua or decidualized tissues. There are gene expression differences between a true decidua elicited by embryo implantation as compared to a deciduoma formed by intrauterine oil injection, intrauterine scratch, or uterine horn crush.

We agree that the term deciduoma more accurately reflects the tissue formed through artificial decidualization methods. We have revised the manuscript to use deciduoma instead of "decidua" or "decidualized tissue."

231 Use the accepted gene nomenclature for estrogen receptor alpha that is ESR1 rather than ER alpha

We agree. We now use "ESR1" instead of "ER alpha" throughout the manuscript. We have also exclusively used the official name "CSPG4" in lieu of "NG2."

225 If a lower number of Tomato+ cells were seeded into the Matrigel, then one would expect lower organoid formation efficiency. Stromal and mesenchymal cells will not survive in Matrigel and the medium used for EMO expansion, thus is the observed outcome simply due to lower numbers of Tomato+ cells being seeded as compared to the control?

We apologize for the confusion. To clarify, both control and Tomato$^+$ organoid cultures were seeded with the same number of cells (40,000 cells). For the control organoids, we used whole endometrial cells (which included some Tomato$^+$ cells) isolated from *Nestin*-CreER; *Rosa*-Tomato uteri 2 days after 4-OHT exposure. For the Tomato-only organoids, we used FACS-purified Tomato$^+$ cells from the same strain and timepoint. Therefore, the observed difference in organoid formation efficiency is not due to differences in initial cell numbers but, instead, likely reflects intrinsic properties of the Tomato$^+$ cell population. In the Methods section of the revised manuscript, we describe the cell numbers used, and we clarified that the same cell number was used for all organoid culture assays (lines 555 and 564).

273 Which ERs? ESR1 or ESR2 or both?

Thank you for the question. "ERs" refers to both ESR1 and ESR2. ICI 182,780 (Fulvestrant), the estrogen receptor antagonist we used, blocks both receptors. We have clarified this point in the revised manuscript in the Results (line 238) and Methods (line 510) sections.

Figure S1B and S1F The CDH1 IF photomicrograph appears out of focus, particularly the higher magnification in S1F of the epithelium.

Thank you for the feedback. We have replaced the IF photomicrographs. We hope the preservation of higher figure resolution during PDF conversion of the revised manuscript will maintain the image quality.

Figure S2 Some of the photomicrographs appear out of focus and blurry.

Thank you for pointing this out. We have replaced the blurry photomicrographs with higher-quality images in the revised manuscript.

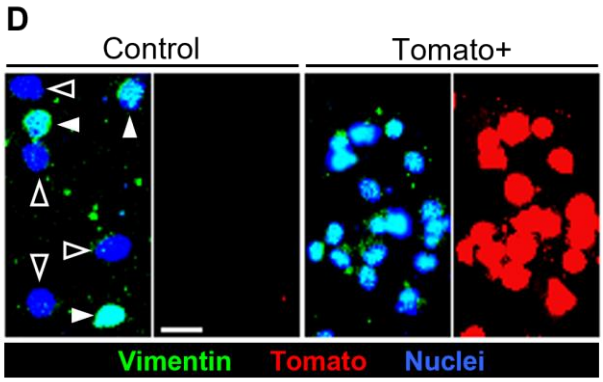
Figure S3A This photomicrograph is of the implantation site, but what happens in the interimplantation sites where the epithelium remains intact and does not have to be regenerated?

The left panel of Supplementary Fig. 3a is an image of the implantation site. The framed area in it focuses on the connecting region between the implantation and inter-implantation site. This framed area highlights the new epithelium which originates from the inter-implantation site to build a new luminal epithelial layer under decidualized stroma in the implantation site. The epithelia of the inter-implantation sites are intact and do not undergo dramatic re-epithelialization, so very few Tomato$^+$ cells are detected.

Figure S4D and S4E The vimentin (VIM) IF is not convincing and appears as background or autofluorescence. The quality of the photomicrographs in S4D is poor and not in focus.

We agree. In the previous Supplementary Fig. 4d we also noticed that Vimentin staining of uterine cells in suspension had some green punctae that are likely background staining or autofluorescence from debris. We prepared a new Supplementary Fig. 4d in which Vimentin staining is robust and we have clearly denoted distinct Vimentin$^+$ and Vimentin$^-$ cells. In the image below, solid arrowheads point to Vimentin$^+$ cells and open arrowheads point to Vimentin$^-$ cells. The staining pattern and subcellular localization may appear unusual, since cell shape changes occur in suspension such that nuclei are tightly surrounded by cytoplasm, and, thus, cells lack the typical filamentous architecture observed in adherent cultures or in vivo that are labeled by Nestin or Vimentin. However, the presence of Vimentin$^+$

and Vimentin^{-} cells in the same sample strongly suggests that staining is not background or autofluorescence. The higher-quality image we now provide should improve clarity.



In Supplementary Fig. 4e we acknowledge that there is some minor background for Vimentin staining, and the faint green signal located next to the Tomato^{+} organoid likely represents non-specific staining and/or debris; at this point, organoids have become epithelial and should no longer express Vimentin.

Figure S4F The ESR1 IF is not convincing. Please show the ESR1 IF channel only independent of DAPI.

We agree. As requested, we have included an image of the ESR1 IF channel alone in Supplementary Fig. 4f.

Reviewer #2 (Remarks to the Author):

The origin of the endometrial epithelium has long been a fundamental question in female reproductive biology. Previous studies suggest that epithelial regeneration arises from multiple distinct sources. This study uncovers an unexpected role of Nestin+ perivascular cells in epithelial regeneration via mesenchymal-to-epithelial transition (MET), using genetic lineage tracing assays in mice. The authors demonstrate that Nestin+ perivascular cells function as progenitor cells, differentiating into epithelial cells to restore the uterine lining after epithelial shedding during the estrous cycle, pregnancy, and artificial decidualization—a model mimicking menstruation.

Mechanistically, this study further explores the molecular pathways regulating this process. The findings reveal that Notch signaling maintains Nestin+ cells in a quiescent state, while estrogen signaling, mediated by estrogen receptor alpha (ERα), suppresses Notch activity to activate these cells and drive their differentiation. Through immunofluorescence imaging, flow cytometry, and quantitative PCR analyses, the study identifies a crucial Estrogen-Notch signaling axis that governs the transition of perivascular cells into epithelial cells.

These findings have significant implications for reproductive health, as impaired endometrial regeneration is associated with conditions such as infertility, endometriosis, and endometrial hyperplasia. By identifying perivascular cells as a novel epithelial progenitor population, this study lays the groundwork for potential therapeutic strategies aimed at enhancing uterine regeneration.

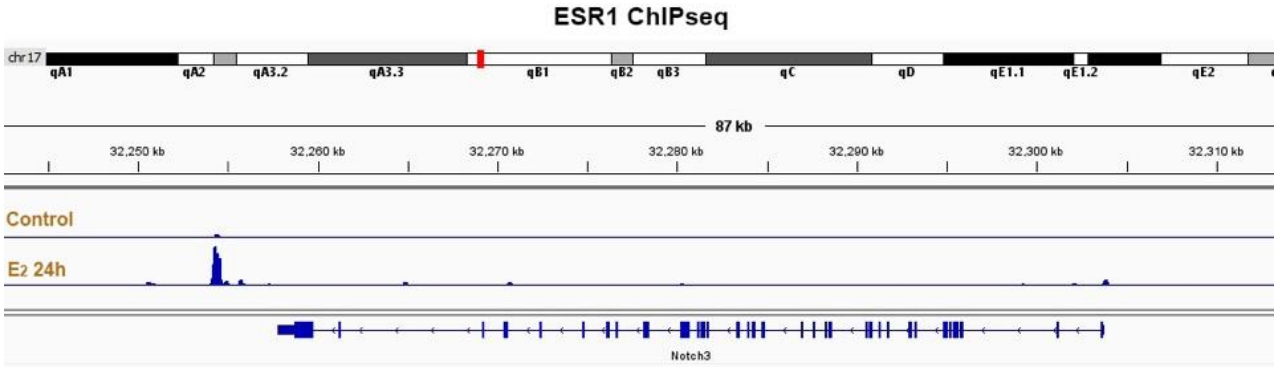
We appreciate the recognition of the novelty and significance of our findings regarding the role of Nestin^{+} perivascular cells in endometrial epithelial regeneration. We have taken care to further clarify and emphasize these points in the revised manuscript.

Minor Comments:
Since Axin2-positive cells have been established as another key source of epithelial regeneration, please provide a more detailed comparison of these two progenitor populations in the discussion section.

We agree that a more detailed comparison between Nestin-positive perivascular cells and Axin2-positive epithelial progenitor cells would enhance the Discussion and offer valuable insight into the distinct roles of these cell populations. We have included this discussion on lines 401-409.

The authors speculate that ERα inhibits the expression of Notch-associated genes. Given that genome-wide ERα distribution data in the uterus is available, is there direct evidence of ERα binding to these genes?

This is an excellent suggestion. Our results in this study suggest that DLL4 from endothelial cells activates Notch3 on perivascular cells. We have analyzed ESR1 ChIP-seq data (GSE36455; PMID: 22446102), and, indeed, there is ESR1 binding to Notch-associated genes. We found an ESR1 binding peak close to *Notch3* upon E2 administration (see image below); however, there was no significant peak detected close to *Dll4*. Interestingly, ESR1 binding peaks were detected near *Notch1*, *-2*, *-3*, and *-4*, suggesting a broad regulatory role for ESR1 on Notch receptor gene expression.



If possible, move Figure S7 to the main figure panel to enhance reader comprehension of the study's findings.

We agree. We have moved Supplementary Fig. 7 into the main figure panel. It is now Fig. 8.

Label Figures 3D, 7A and S2B to clearly distinguish the two rectangles.

Thank you for pointing this out. As requested, we have revised Figs. 3d, 7a, and Supplementary Fig. 2b.

Reviewer #3 (Remarks to the Author):

The manuscript by Li et al. presents evidence that Nestin-positive perivascular cells participate in endometrial epithelial homeostasis and regeneration through mesenchymal-to-epithelial transition. By

using mouse genetic models and both in vitro and in vivo lineage-tracing approaches, they convincingly demonstrate an additional epithelial progenitor cell source beyond previously characterized basal epithelial and stromal populations. These insights advance our understanding of re-epithelialization in the endometrium and underscore how perivascular cell plasticity may contribute to tissue repair following natural or induced endometrial shedding. Although this is a scientifically sound manuscript, I have several concerns, that if addressed, will strengthen the manuscript

We sincerely thank the reviewer for their positive evaluation of our study and for recognizing the novelty and significance of our findings regarding Nestin-positive perivascular cells in endometrial epithelial regeneration. We have carefully considered the points brought up and have addressed each of them in the revised manuscript to further strengthen the clarity and rigor of our findings.

1. While the authors explore a role for estrogen in activating perivascular cells, studies in rodents and primates have demonstrated that endometrial repair occurs independently of sex hormones (Hartman 1944; Tu'uhevaha J Kaitu'u-Lino , et al. 2007; Kaitu'u-Lino et al. 2010). The authors should acknowledge these findings and more directly discuss how hormone dispensability or context-specific hormone requirements might intersect with their proposed mechanism of Nestin-positive perivascular cell–driven re-epithelialization.

We agree and have now included these studies in the Discussion section where we describe hormone-independent endometrial repair (lines 448-454).

2. Given that a core aspect of this study hinges on microscopic evaluation of lineage-traced cells, higher-resolution, more clearly labeled images would provide stronger evidence. This issue may have been partially impact due to image size restrictions at submisiion

We have updated the figures in the revised manuscript with higher-resolution images.

3. In line 170, the authors state, "Interestingly, we also observed a Tomato+ cell half-embedded in the epithelial layer, suggesting it was undergoing a transition." It is unclear whether this is a routine observation or if it is a rare observatiion. Providing quantitative data (would be valuable. In addition, clarifying whether these cells show any known MET markers would solidify the claim that they are actively transitioning to an epithelial phenotype.

This is a valid point. Due to the transient nature of the transition process and the relatively low abundance of Nestin-derived epithelial cells, it is difficult to capture intermediate states. We have clarified this point in the Results section of the revised manuscript (lines 154-157).

4. The authors mainly rely on immunofluorescence to assess the presence and number of Nestin-derived cells, particularly where the overall numbers appear low. While this is acceptable in some instances, flow cytometry would offer a more robust and quantitative measure of these rare populations.

While we agree that flow cytometry could provide a more quantitative assessment of rare populations, we chose to rely on immunofluorescence due to its ability to preserve spatial context and tissue architecture, so that we can definitively identify the location(s) and types of cells of interest. We feel that imaging analyses also allow us to observe cell clones/clusters and the nature of Nestin-derived cell contribution to the epithelium. However, we did use flow cytometry in Fig. 1h to show that the initial

Tomato+ population was not epithelial and in Supplementary Fig. 1c to quantify Nestin$^+$ cells in the uterus.

5. The methods for organoid establishment need to be expanded, particularly regarding cell seeding density. Differing numbers of plated cells across experimental conditions could confound comparisons of organoid-forming efficiency.

We appreciate the reviewer's suggestion. In this study, cell seeding density was carefully controlled to ensure consistency across experimental conditions. Specifically, a total of 40,000 whole endometrial cells or FACS-purified Tomato$^+$ cells were embedded in 30 µL of 70% Matrigel for each organoid culture condition. We have clarified the protocol used for organoid assays in the Methods section, and we have emphasized that the same number of cells was seeded for each organoid culture assay (lines 555 and 564).

6. The presence of tdTomato-positive cells in "control" organoids needs further clarification. It would be helpful to confirm whether any tamoxifen exposure or lineage-leakage effects led to unexpected labeling, and to elaborate on how promoter strength variations might explain delayed or minimal epithelial labeling. In addition, negative selection of CDH1 or EPCAM before plating tdTomato-positive perivascular cells would provide a cleaner starting population, allowing stronger conclusions about perivascular to epithelial transition in vitro.

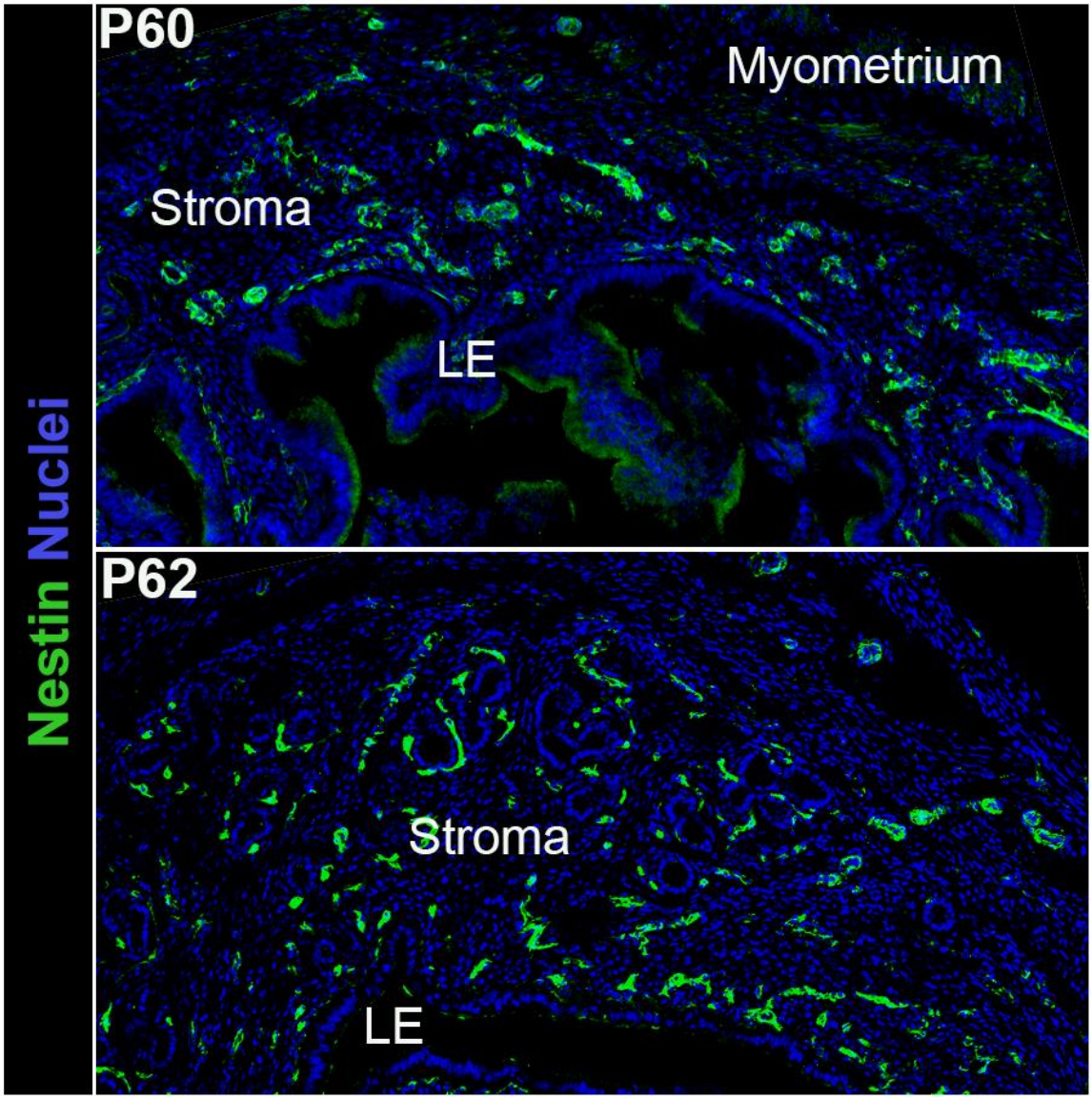
We apologize for the lack of clarity in our description of "control" organoids. Control organoids were generated using whole endometrial cells from *Nestin*-CreER; *Rosa*-Tomato uteri 2 days post 4-OHT exposure; therefore, there was a small percentage of Tomato$^+$ cells in the initial pool of cells used to seed the organoids. Despite these control samples containing some Tomato$^+$ perivascular cells, the pre-existing epithelial cells are more adept or efficient at organoid formation as compared to all other cells, so that is likely the reason why we did not observe Tomato$^+$ cells in organoids under these conditions. When using purified Tomato$^+$ cells only, we readily observed their ability to self-organize into organoids. Another possible explanation is that, in conditions with purified Tomato$^+$ cells, the chance for Tomato$^+$ cells interacting with other Tomato$^+$ cells is high, which initiates the aggregation of Tomato$^+$ cells into organoids. In the control group, Tomato$^+$ cells are surrounded by various Tomato-negative cell types, which prevents the aggregation of Tomato$^+$ cells. Epithelial cells (which are Tomato-negative) can easily aggregate to form organoids without the need of cell transformation. Although it is possible that isolated Tomato$^+$ cells can survive in Matrigel or even undergo MET in the control assays, it is too late to join the organoids that are formed more rapidly by preexisting Tomato-negative epithelial cells.

Given the absence of Tomato$^+$ cells in control organoids with cells from un-purified whole tissue, we did not attempt to culture organoids using purified Tomato-negative cells, as it would be redundant.

7. The authors should address potential confounding influences of tamoxifen treatment itself on Nestin expression or on the uterus more broadly. Although the authors have taken precautions to mitigate tamoxifen's antiestrogenic effects, validating that Nestin expression in the uterine epithelium is not an artifact of tamoxifen treatment is essential. This could be done by qRT-PCR or ISH.

We completely agree that it is important to rule out potential confounding effects of tamoxifen treatment on Nestin expression. To address this issue, we used immunofluorescence to compare the expression of Nestin before (at P60) and immediately after (at P62) two tamoxifen injections on P60 and P61. There is

no significant difference in the intensity, density, or pattern of Nestin expression before and after the induction. The thickness of the stromal layer is increased after tamoxifen injections, since overall uterine size is increased post-tamoxifen-injection on P62, but given the close interaction between perivascular and endothelial cells, it is not unexpected to see a fluctuation of perivascular cell numbers with changes in uterine vasculature. The most important point is that only perivascular cells, but no epithelial cells, were labeled with Tomato immediately after tamoxifen induction (Fig. 1c, d, and h). This is the foundation to support all our subsequent MET observations. However, to reinforce this point further, we have provided new images showing that Nestin expression in luminal epithelial (LE) cells is not observed after tamoxifen injection). However, since this data is largely redundant with Fig. 1a and 1b, which also show Nestin expression, we did not include it in the revised version of the manuscript. If the reviewer thinks this data is helpful for the reader, we are willing to add it to a supplemental figure.



We are grateful to the reviewers for their helpful comments and critiques. Thank you very much for your consideration.

Sincerely,

Tony DeFalco, Ph.D.
Associate Professor, Division of Developmental Biology
Co-Director, Reproductive Sciences Center
Cincinnati Children's Hospital Medical Center
Department of Pediatrics
University of Cincinnati College of Medicine

Reproductive Sciences Center
Division of Developmental Biology

Tony DeFalco, Ph.D.
Associate Professor
Co-Director

Location S, Room 216
3333 Burnet Avenue, MLC 7045
Cincinnati, OH 45229-3039
Phone: (513) 803-3988
Fax: (513) 803-1160
Email: Tony.DeFalco@cchmc.org

August 5, 2025

RE: Manuscript ID #NCOMMS-25-10067A

Dear Reviewers,

Reviewer comments are in black font and our responses are listed below in blue font.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

SUMMARY

This manuscript utilizes a genetic lineage tracing model to investigate novel mechanisms of epithelial remodeling and repair in the mouse uterus. The results support the idea that Nestin+ perivascular cells can be activated under certain conditions to differentiate into epithelial cells.

OVERALL AND MAJOR COMMENTS

The revised manuscript is improved and has addressed many of the major and minor comments based on review of the initial manuscript.

Major Comments:

1) Endometrial regeneration after parturition occurs in the absence of estrogen, thus estrogen is not always the driver of endometrial regeneration.

We agree with the reviewer's comment that estrogen is not strictly required for endometrial regeneration, and we have stated that this process is hormone-independent (Discussion, lines 449-452). We think epithelial repair involving perivascular cells/pericytes is an additional mechanism that supports the major mechanism of repair using preexisting epithelial cells, and estrogen plays a key role in pericyte activation. We have clarified and emphasized these points in the Discussion on lines 440-454.

2) Figures (Main and Supplementary): The major cell types in one or more panels of the figures need to be clearly labeled in the sections to enhance interpretation and readability. For instance, LE, GE, Stroma, etc. This is correctly done in Supplementary Figure 2.

We agree that labeling of major cell types would be informative. We have added new labels to denote major cell types in Figures 1-7 and Supplementary Figures 1, 3, and 6.

3) Figures: Please indicate in figure legend or text the type of section used for immunolocalization such as transverse or longitudinal.

As requested, we have added information about the type of section used, i.e., cross-sectional vs. longitudinal, for immunofluorescence analyses in the corresponding figure legends (Figures 1-7 and Supplementary Figures 1, 2, 3, and 6).

4) Figure 5: The higher magnification photomicrographs in Panel a (middle and right) are not in focus and blurry.

We agree. We have replaced those 2 images in Figure 5a with newer images that are of higher quality.

SPECIFIC COMMENTS

Line Comment

22 The epithelial cells undergo apoptosis during the estrous cycle…they are not shed.

As suggested, we changed the text to state that epithelial cells undergo apoptosis during estrous cycles (line 22).

37 see above comment

We have changed the text to state that cells undergo apoptosis during mouse estrous cycles and that the process in mice is distinct from humans (lines 36-39).

78 Please use only the accepted gene nomenclature of ESR1

We now use only ESR1 throughout the manuscript (lines 29 and 78).

108 stained should be immunostained

We have changed this word to “immunostained” (line 108).

138 “antimesometrial stroma” not stroma of the anti-mesometrium

We have modified the text to state “antimesometrial stroma” (line 138).

364 Add “potentially” before contribute

We have added the word “potentially” here (line 363).

453 Avoid using theatrical terms such as “drastic” and “dramatic”

We have replaced these terms with the more neutral term “significant” (lines 388 and 453).

Reviewer #2 (Remarks to the Author):

The response addresses all of my concerns—no further questions.

We thank the reviewer for their feedback on our manuscript.

Reviewer #3 (Remarks to the Author):

The authors have adequately addressed my comments.

We thank the reviewer for their feedback on our manuscript.

We are grateful to the reviewers for their helpful comments and critiques. Thank you very much for your consideration.

Sincerely,

Tony DeFalco, Ph.D.
Associate Professor, Division of Developmental Biology
Co-Director, Reproductive Sciences Center
Cincinnati Children’s Hospital Medical Center
Department of Pediatrics
University of Cincinnati College of Medicine