EMBO reports

Retinoblastoma protein promotes uterine epithelial cell cycle arrest and necroptosis for embryo invasion

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 25th Jun 2020

Dear Dr. Hirota

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting. However, both referees also point out several technical concerns and have a number of suggestions for how the study should be strengthened, and I think that all of them should be addressed.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We invite you to submit your manuscript within three months of a request for revision. This would be September 25th in your case. Yet, given the current COVID-19 related lockdowns of laboratories, we have extended the revision time for all research manuscripts under our scooping protection to allow for the extra time required to address essential experimental issues. Please contact us to discuss the time needed and the revisions further.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section is missing.
2) Your manuscript contains error bars based on n=2. Please use scatter blots showing the individual datapoints in these cases. The use of statistical tests needs to be justified.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages
https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines
()

6) Supplementary Information: Please note that a maximum of 5 Expanded View Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content incl. page numbers. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Please note that a Data Availability section at the end of Materials and Methods is now mandatory. In case you have no data that requires deposition in a public database, please state so instead of referring to the database.
See also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>).
Please note that the Data Availability Section is restricted to new primary data that are part of this study.

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available .

9) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

10) Regarding data quantification:

- Please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
IMPORTANT: Please note that error bars and statistical comparisons may only be applied to data obtained from at least three independent biological replicates. If the data rely on a smaller number of replicates, scatter blots showing individual data points are recommended.
- Graphs must include a description of the bars and the error bars (s.d., s.e.m.).
- Please also include scale bars in all microscopy images.

11) As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely

Martina Rembold, PhD
Editor
EMBO reports

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Referee #1:

The manuscript "Uterine retinoblastoma stimulates epithelial cell cycle arrest and necroptosis for embryo invasion" provides evidence for an involvement of the "uterine" Retinoblastoma gene and P4 in embryo invasion. The study was well-designed and conducted and conclusions are supported by the data. However, there are some points that should be addressed to clarify the usage of the transgenic mouse models and to improve the structure and clarity of the manuscript.
Abstract:
-Please better define the connection between Rb1 and P4. Do both CCA inducers directly promote each other meaning does Rb1 induce P4 or vice versa?
Introduction:
-page 5, lines 18-19: The sense of this sentence remains unclear. Did the authors aim to study whether epithelial CCA is important for embryo invasion? As stated before (lines 12-13) epithelial

CCA seems to be a hallmark of uterine receptivity and the authors rather aimed to analyze the underlying mechanism of this process. Please comment on this.
-page 6, lines 9-11: The objectives of this study are ill-defined. Please better define the aims of the study including Rb1, P4 and CCA.
Results:
-results section contains several general explanations and conclusions that prolongs this section dramatically. Thus, authors should think about transferring general explanations into the introduction section and conclusions into the discussion section.
-page 7, lines 4-7: It seems that pRb expression is limited to 2 days before implantation takes place. Does this mean that Rb activity is reduced at these two days? If this is the case, conclusions about the activity of uterine Rb should be drawn more precisely than just stating "uterine Rb is active in peri-implantation period".
page 7, lines 8-9: Did the authors check for Rb deletion in other tissues than uterus? Is there any chance that Rb deletion in tissues besides the uterus (e.g. ovary) will affect pregnancy outcome?
-page 9, line 16: Rb deletion seems to provoke an implantation arrest rather than a complete implantation failure as embryo attachment worked properly. Please comment on this.
Discussion:
-page 20, lines 11-14: The reviewer kindly asks the authors to be more precise in their explanation on how uterine stromal cells regulate detachment of the luminal epithelium
-page 22, lines 8-14: The reviewer wonders why the authors limit their concluding remarks on P4 as during their study they provided plenty of evidence for a function of Rb in embryo invasion.
Materials and Methods:
-page 23, lines 5-8: As Pgr is widely expressed in several tissues besides the uterus, the reviewer doubts that Pgr-Cre mice have specific expression of the Cre recombinase in the uterine epithelium and kindly asks the authors to prove specific expression. Same for Ltf-Cre mice. Is lactotransferrin specifically expressed in uterine epithelium during pregnancy?
-page 24, line 6: What was the rationale behind injecting the dose of 2mg P4?
-page 24, lines 8-13: Did all patients receive P4 in the ET cycles or were some embryos transferred during natural menstrual cycles without hormonal supplementation? The reviewer wonders how P4 treatment in the cycles where endometrial biopsies have been taken can have such a long-term effect on pregnancy outcome in the following cycles?

Referee #2:

It's an interesting story and does add new insight in identifying cell cycle arrest in the epithelium as a permissive step in implantation in the mouse. It is particularly interesting that embryo attachment, and signalling to increase vascular permeability and initiate decidualisation, both occur in Rb1 nulls, but implantation progression is blocked, perhaps (they speculate) because of incomplete engagement with the stroma. Or is there adverse inflammatory activity as a result of the persistence of epithelium?

The title should be changed as it could imply that this is a paper on a rare tumour.

I am not clear how P supplementation overcomes the KO phenotype, given that ovarian function is not affected. Can the authors provide data to throw light on this?

Late in the results, data are revealed that suggest the Rb1 KO epithelial phenotype (inability to be

phagocytosed by TGC) results from a block in paracrine signalling from stroma to epithelium. This could be followed up using other KO models but would require substantially more work, so I am not requesting it. But this important finding should be represented better in the discussion, with reference to what is known about P-dependent stromal-epithelial signalling.

Necroptosis: RIP1/3 is acceptable, but can this mechanism of cell death be confirmed using morphological markers, as neither lipid droplets nor PS exposure are specific? Is there evidence that caspases are not activated? There seems to be little change to the epithelial nuclear morphology. Based on the TEM in Figure EV2, can the authors definitively exclude entosis?

ECC1 is a poor choice of cell line for human relevance because although it was published as an endometrial line it was subsequently shown to be a strain of HeLa. Please repeat using a bona fide endometrial line.

p13 line 13 'recovered resorption' is not clear, I think it should be eg 'rescued the implantation sites'

Insufficient data are provided about the human tissues. How many biopsies? How many became pregnant? There may be differences between cycles in the same woman. We need to see the distribution of Ki67+ index on normal day LH+7 as a baseline before any conclusion can be drawn about those who did not conceive.

Statistics: the statement that 'P<.05 was considered statistically significant' is no longer acceptable. Were the data normally distributed? Data should be shown as a dot plot so one can see the variation. An appropriate test should be conducted and the actual P value given together with a measure of variance. There is no need to employ the term 'significant'.

August 30, 2020

Dr. Martina Rembold, PhD
Editor, EMBO Reports

RE: EMBOR-2020-50927V1

Dear Dr. Rembold:

My co-authors join me in expressing our sincere appreciation to you and reviewers for your efficient handling of the manuscript and thoughtful comments. Each reviewer has been very helpful and constructive to improve our manuscript. We have critically reviewed each comment and our responses are elaborated below. The editor's and reviewers' comments are followed by our responses highlighted in **blue**.

Editor:

As you will see, the referees acknowledge that the findings are potentially interesting. However, both referees also point out several technical concerns and have a number of suggestions for how the study should be strengthened, and I think that all of them should be addressed. Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We appreciate these comments. We have performed additional experiments and edited the revised manuscripts to address the reviewers' concerns. Our point-by-point responses are described below. Please reconsider our revised form of the manuscript for publication in EMBO Reports.

Referee #1:

The manuscript "Uterine retinoblastoma stimulates epithelial cell cycle arrest and necroptosis for embryo invasion" provides evidence for an involvement of the

"uterine" Retinoblastoma gene and P4 in embryo invasion. The study was well-designed and conducted and conclusions are supported by the data. However, there are some points that should be addressed to clarify the usage of the transgenic mouse models and to improve the structure and clarity of the manuscript.

We appreciate the constructive comments of this reviewer. According to the comments, we have revised the manuscript. Our point-by-point responses are described below.

1. Abstract:

-Please better define the connection between Rb1 and P4. Do both CCA inducers directly promote each other meaning does Rb1 induce P4 or vice versa?

We appreciate this comment. In the revised abstract, we have described that retinoblastoma protein (RB) encoded by *Rb1* induces cell cycle arrest (CCA), and progesterone (P_4) promotes CCA in uterine epithelium, and P_4 activates RB by reducing the phosphorylated RB (pRB), the inactivated form of RB, in uterine epithelium (**Page 3, Lines 3-5**). In addition, we have described in the introduction section that estrogen increases pRB and promotes cell proliferation in the uterine epithelium, which is suppressed by P_4, indicating that uterine RB controls ovarian hormone-dependent cell proliferation status (**Chen B, et al. Mol Endocrinol 19, 1978-90, 2005; Tong W, et al. Mol Cell Biol 19,2251-64, 1999**) (**Page 6, Lines 16-19**). These descriptions can enable the readers to understand the connection between RB and P_4.

2. Introduction:

-page 5, lines 18-19: The sense of this sentence remains unclear. Did the authors aim to study whether epithelial CCA is important for embryo invasion? As stated before (lines 12-13) epithelial CCA seems to be a hallmark of uterine receptivity and the authors rather aimed to analyze the underlying mechanism of this process. Please comment on this.

We agree to this comment. As the reviewer pointed out, we aimed to analyze the underlying mechanism of the process of embryo implantation. We have described in the introduction section that this study is aimed to analyze the underlying mechanisms of embryo implantation by focusing on CCA regulators in the uterus (**Page 6, Lines 4-6**).

3. -page 6, lines 9-11: The objectives of this study are ill-defined. Please better

define the aims of the study including Rb1, P4 and CCA.

We agreed to this comment. According to the reviewer's comment, we have described the aims of the study in the introduction section as shown below.

"In the present study using female mice with uterine *Rb1* deficiency (*Rb1*$^{d/d}$ mice), we aimed to investigate the fundamental machinery in which uterine RB and P_4 are involved in embryo implantation through epithelial CCA." (**Page 6, Line 19-Page 7, Line 2**).

4. Results:

-results section contains several general explanations and conclusions that prolongs this section dramatically. Thus, authors should think about transferring general explanations into the introduction section and conclusions into the discussion section.

We appreciate this comment. We have transferred general explanations from the Results section to the Introduction section and the Discussion section.

5. -page 7, lines 4-7: It seems that pRb expression is limited to 2 days before implantation takes place. Does this mean that Rb activity is reduced at these two days? If this is the case, conclusions about the activity of uterine Rb should be drawn more precisely than just stating "uterine Rb is active in peri-implantation period".

We appreciate this comment. We have included the following precise description in the revised manuscript.

"In contrast, phosphorylated RB (pRB), the inactive form of RB, is expressed in uterine epithelium on days 2 and 3 but not on day 4, suggesting that the suppression of RB activity is lost on day 4. These findings indicate that uterine RB becomes functionally active on day 4 when uterine PDS takes place." (**Page 8, Lines 4-8**).

6. -page 7, lines 8-9: Did the authors check for Rb deletion in other tissues than uterus? Is there any chance that Rb deletion in tissues besides the uterus (e.g. ovary) will affect pregnancy outcome?

We appreciate this comment. Since Cre recombinase is expressed in the ovary in *Pgr*-Cre mice, we have examined the expression of RB in the pregnant ovary in *Rb1*$^{d/d}$ mice. We found that RB is normally expressed in the ovarian granulosa and lutein cells of *Rb1*$^{d/d}$ mice (**Fig 1D; Page 8, Lines11-12**), suggesting that RB is not deleted in the ovary of *Rb1*$^{d/d}$ mice. Nonetheless, we have examined ovulation, fertilization,

pre-implantation embryonic growth and ovarian hormonal secretion as ovarian functions and found that all the processes are normal in *Rb1*$^{d/d}$ mice (**Fig 2A-C & 4A-B**). These findings indicate that the reproductive phenotypes of *Rb1*$^{d/d}$ mice are due to uterine *Rb1* deletion. We have described this information in the revised Results section and Materials & Methods section (**Page 12, Lines 10-11; Page 24, Lines 5-13**).

7. -page 9, line 16: Rb deletion seems to provoke an implantation arrest rather than a complete implantation failure as embryo attachment worked properly. Please comment on this.

We appreciate this comment. According to this comment, we have described particularly in the revised manuscript that embryo implantation failed after normal embryo attachment in *Rb1*$^{d/d}$ mice (**Page 10, Lines 3-4**).

8. Discussion:

-page 20, lines 11-14: The reviewer kindly asks the authors to be more precise in their explanation on how uterine stromal cells regulate detachment of the luminal epithelium

We appreciate this comment. According to the reviewer's comment, we have included the detailed description of luminal epithelium detachment regulated by stromal HIF2α in the revised Discussion section (**Page 21, Lines 13-17**).

9. -page 22, lines 8-14: The reviewer wonders why the authors limit their concluding remarks on P4 as during their study they provided plenty of evidence for a function of Rb in embryo invasion.

We appreciate this comment. According to the reviewer's comment, we have described the concluding remarks on the functions of not only P_4 but RB in embryo implantation (**Page 23, Lines 16-19**).

10. Materials and Methods:

-page 23, lines 5-8: As Pgr is widely expressed in several tissues besides the uterus, the reviewer doubts that Pgr-Cre mice have specific expression of the Cre recombinase in the uterine epithelium and kindly asks the authors to prove specific expression. Same for Ltf-Cre mice. Is lactotransferrin specifically expressed in uterine epithelium during pregnancy?

We appreciate this comment. In *Ltf*-Cre female mice, Cre recombinase is specifically

expressed in the uterine epithelium (**Daikoku T, et al. Endocrinology 155, 2718-24, 2014**). In the previous and current studies, the floxed genes *Hif2a* and *Rb1* were specifically deleted in the uterine epithelium of the pregnant mouse models using *Ltf*-Cre female mice (**Matsumoto L, et al. JCI 128, 3186-97, 2018**) (**Fig 8A**). In *Pgr*-Cre female mice, Cre recombinase is expressed not only in the uterus but in the ovary (**Soyal SM, et al. Genesis 41, 58-66, 2005**). Although *Pgr*-Cre mouse line can be used for deletion of the floxed gene in the entire uterus (**Matsumoto L, et al. JCI 128, 3186-97, 2018**), the events regulated by ovarian functions such as ovulation and fertilization need to be evaluated in the pregnant mouse models using *Pgr*-Cre females (**Haraguchi H, et al. FASEB J 33, 2610-20, 2019**). We have investigated ovulation, fertilization, pre-implantation embryonic growth and ovarian hormonal secretion, and found that all the physiological processes are normal in $Rb1^{d/d}$ mice (**Fig 2A-C & 4A-B**). In addition, we have examined the expression of RB in the pregnant ovary as well as the uterus in $Rb1^{d/d}$ mice, and found that RB is efficiently deleted in the pregnant uterus (**Fig 1C**) but is normally expressed in the ovarian granulosa and lutein cells of $Rb1^{d/d}$ mice (**Fig 1D**). These findings indicate that the reproductive phenotypes of $Rb1^{d/d}$ mice are due to uterine *Rb1* deletion. We have included the description about this information in the revised manuscript (**Page 12, Lines 10-11; Page 24, Lines 5-13**).

11. -page 24, line 6: What was the rationale behind injecting the dose of 2mg P4?

We appreciate this comment. Daily P_4 injection at the dose of 2mg/day/mouse was used for the induction of embryo implantation in the delayed implantation mouse model (**Yoshinaga K, Adams CE. J Reprod Fertil 12,593-5,1966; Huet YM, Dey SK. J Reprod Fertil 81, 453-8, 1987; Ma W, Song H, et al. PNAS 100, 2963-8, 2003**). In our previous study, we confirmed that daily administration of P_4 at the dose of 2 mg/day/mouse from day 2 is not harmful for embryo implantation in wild-type mice (**Matsuo M, Hirota Y, et al. Endocrinology 161, bqz005, 2020.**). We have included this information in the revised Materials & Methods section (**Page 25, Lines 7-11**).

12. -page 24, lines 8-13: Did all patients receive P4 in the ET cycles or were some embryos transferred during natural menstrual cycles without hormonal supplementation? The reviewer wonders how P4 treatment in the cycles where endometrial biopsies have been taken can have such a long-term effect on pregnancy outcome in the following cycles?

We appreciate this comment. Both endometrial biopsy and embryo transfer were

performed in the artificial cycle. We confined the endometrial samples to those in the artificial cycle to minimize the difference of hormonal conditions among the samples. We did not expect to see the long-term effect of P_4 supplementation in the endometrial biopsy cycle. As the limitation of the experiments using human subjects, we substituted the endometria in the endometrial biopsy cycle prior to the embryo transfer cycle for those in the embryo transfer cycle. We have included the above information in the revised Materials & Methods section (**Page 25, Line 13-Page 26, Line 8**).

"As the limitation of the experiments using human subjects, it is practically impossible to perform human endometrial biopsy in the implantation period of the embryo transfer cycle. Since the endometrial genes related to uterine receptivity are expressed in a reproducible fashion among the cycles (**Diaz-Gimeno P, et al. Fertil Steril 99, 508-17, 2013**), we consider that the uterine receptivity-associated uterine cell proliferation status in the cycle prior to the embryo transfer cycle corresponds to that in embryo transfer cycle. Thus, we substituted the endometria in the cycle prior to embryo transfer instead. Endometrial biopsy was performed at the 7th day of P_4 treatment in an artificial cycle which is considered as the implantation period in humans. To minimize the differences of hormonal conditions among the individual patients, the same protocol of artificial hormonal treatment in the cycle of endometrial biopsy was used for all the patients. The patients underwent embryo transfer in the following artificial cycles after endometrial biopsy. We investigated the relationship between the peri-implantation uterine cell proliferation status and the pregnancy outcome. The samples were divided into two groups according to the outcome of clinical pregnancy following endometrial biopsy."

Referee #2:

It's an interesting story and does add new insight in identifying cell cycle arrest in the epithelium as a permissive step in implantation in the mouse.

We are pleased with the constructive comments of this reviewer. Our responses to the comments are addressed below.

1. It is particularly interesting that embryo attachment, and signalling to increase vascular permeability and initiate decidualisation, both occur in Rb1 nulls, but implantation progression is blocked, perhaps (they speculate) because of incomplete engagement with the stroma. Or is there adverse inflammatory activity as a result of the persistence of epithelium?

We appreciate this comment. *Rb1* eKO mice (uterine epithelial *Rb1* deficient mice) showed normal epithelial CCA and embryo invasion in spite of effective deletion of epithelial RB, speculating that stromal RB is involved in epithelial CCA and embryo invasion. GO analysis demonstrated that the gene cluster in which the transcripts were downregulated in the luminal epithelium of $Rb1^{d/d}$ mice without P_4 treatment compared to other groups was not related to inflammation but to epithelial differentiation and inhibition of cell proliferation (**Fig EV5C**), suggesting the pathological processes other than inflammation in the luminal epithelium may cause embryo invasion failure in $Rb1^{d/d}$ mice. We have described this speculation in the Discussion section (**Page 21, Line 20-Page 22, Line 5**).

2. The title should be changed as it could imply that this is a paper on a rare tumour.

We appreciate this comment. According to this comment, we have changed the title to "**Uterine retinoblastoma protein stimulates epithelial cell cycle arrest and necroptosis for embryo invasion**".

3. I am not clear how P supplementation overcomes the KO phenotype, given that ovarian function is not affected. Can the authors provide data to throw light on this?

We appreciate this comment. To examine the influence of *Rb1* and P_4 on transcriptome in the luminal epithelium, we performed RNA-seq using day 4 luminal epithelium dissected out from $Rb1^{f/f}$ mice and $Rb1^{d/d}$ mice with and without P_4 supplementation by laser capture microdissection. We found 2185 differentially expressed genes (DEGs) in at least one group compared to others (**Fig EV5A**). Among 10 unique clusters defined by K-means clustering, we focused on the gene cluster in which the transcripts are poorly expressed in $Rb1^{d/d}$ mice without P_4 supplementation compared to other groups. We examined the profile of the genes belonging to this cluster using publicly available database of RNA-seq and ChIP-seq regarding transcriptional factors (TFs). We found that DEGs in this cluster are poorly correlated to PGR but highly correlated to cell cycle-related TFs (**Fig EV5B**). In addition, gene ontology (GO) analyses also revealed that this cluster is related to epithelial differentiation and inhibition of cell proliferation (**Fig EV5C**). These results indicate that P_4 administration to $Rb1^{d/d}$ mice rescues embryo invasion failure by specifically influencing the cell proliferation pathway of the luminal epithelium. We have included these data in the revised manuscript (**Page 16, Line 13-Page 17, Line**

6).

4. Late in the results, data are revealed that suggest the Rb1 KO epithelial phenotype (inability to be phagocytosed by TGC) results from a block in paracrine signalling from stroma to epithelium. This could be followed up using other KO models but would require substantially more work, so I am not requesting it. But this important finding should be represented better in the discussion, with reference to what is known about P-dependent stromal-epithelial signalling.

We appreciate this comment. We have referred to the previous study (**Li Q, et al. Science 331, 912-6, 2011**) and added the description about P_4-dependent signaling from stroma to epithelium through stromal *Hand2* in the revised Discussion section. Uterine stromal-epithelial communication contributes to successful embryo implantation. The basic helix-loop-helix transcriptional factor *Hand2* in the uterine stroma suppresses the production of fibroblast growth factors that act as paracrine inducers of estrogen-dependent epithelial proliferation (**Page 21, Lines 6-11**).

5. Necroptosis: RIP1/3 is acceptable, but can this mechanism of cell death be confirmed using morphological markers, as neither lipid droplets nor PS exposure are specific? Is there evidence that caspases are not activated? There seems to be little change to the epithelial nuclear morphology. Based on the TEM in Figure EV2, can the authors definitively exclude entosis?

We appreciate this comment. The present study revealed that at the lateral sides of the implantation chamber, PS is exposed at the outer membranes of luminal epithelium, pRIP3 is expressed in the cytoplasm of luminal epithelium and dying epithelial cells are engulfed by the neighboring trophoblast cells. It has been reported that PS exposure is observed in not only apoptotic but non-apoptotic cell death including necroptosis and entosis, and pRIP3 is a specific marker of necroptosis, and phagocytes engulf live cells in the process of entosis and dying cells in the processes of apoptosis and necroptosis (**Tang D, et al. Cell Res 29, 347-364, 2019; Shlomovitz I, et al. Cell Commun Signal 17, 139, 2019; Segawa K, et al. PNAS 115, 12212-17, 2018**). Our findings indicate that necroptosis is involved in luminal epithelium elimination at the lateral sides of the implantation chamber during the initial disappearance of luminal epithelium, which is a similar physiological phenomenon to synaptic pruning. Given that epithelial entosis occurs at the lateral sides of the implantation chamber where epithelial necroptosis takes place, at least it is certain that

non-apoptotic cell death mechanisms including necroptosis and entosis play major roles in the initial step of luminal epithelium barrier removal during embryo invasion. We have included these discussions in the revised Discussion section (**Page 20, Line 7-Page 21, Line 1**).

6. ECC1 is a poor choice of cell line for human relevance because although it was published as an endometrial line it was subsequently shown to be a strain of HeLa. Please repeat using a bona fide endometrial line.

We appreciate this comment. In the revised manuscript, we have shown the data using HEC151 cell line, an endometrial epithelial cell line in the revised **Fig EV4B-D**. The results of the experiments using of HEC151 cells were similar to those using primary mouse epithelial cells.

7. p13 line 13 'recovered resorption' is not clear, I think it should be eg 'rescued the implantation sites'

We appreciate this comment. We have replaced 'recovered resorption' with 'rescued the implantation sites' in the revised manuscript (**Page 13, Line 13**).

8. Insufficient data are provided about the human tissues. How many biopsies? How many became pregnant? There may be differences between cycles in the same woman. We need to see the distribution of Ki67+ index on normal day LH+7 as a baseline before any conclusion can be drawn about those who did not conceive.

We agree to this comment. We have replaced bar graphs by scatter blots in the revised **Figure 8B**. We have clearly described the number of the endometrial samples in pregnant and non-pregnant groups in the revised figure legend (n=10 different individual sample in each group; **Page 44, Line 21**). Three different high-powered fields per sample were analyzed and each of the Ki67-posivie ratios was demonstrated in the revised **Figure 8B**. As the limitation of this study, it was practically difficult to perform repeated endometrial biopsy in the same patients. To minimize the differences of hormonal conditions among the individual patients, the same protocol of artificial hormonal treatment in the cycle of endometrial biopsy was used for all the patients. We have included this description in the revised Materials & Methods section (**Page 25, Line 13-Page 26, Line 8**).

9. Statistics: the statement that 'P<.05 was considered statistically significant' is

no longer acceptable. Were the data normally distributed? Data should be shown as a dot plot so one can see the variation. An appropriate test should be conducted and the actual P value given together with a measure of variance. There is no need to employ the term 'significant'.

We appreciate these comments. According to the reviewer's comments, we have demonstrated scatter blots throughout the revised figures to understand the variation of data easily. We have omitted the term 'significant' throughout the manuscript.

Again, we sincerely appreciate your efficient handling of the manuscript and useful comments of the reviewers to improve the quality of the manuscript. We hope that we have improved this manuscript by taking the editor's and reviewers' comments into consideration.

1st Revision - Editorial Decision 17th Nov 2020

Dear Dr. Hirota

Thank you for the submission of your revised manuscript to EMBO reports. I apologize for my delayed response. Referee #1 was not available anymore but we have now received the report from former referee #2 (copied below), who supports publication without further revision.

Browsing through the manuscript myself, I noticed a few editorial things that we need before we can proceed with the official acceptance of your study.

- I attach to this email two related manuscript files with comments by our data editors in the figure legends. Please address all comments. Note that the comments had been added to an earlier version of your manuscript before you made further changes to the manuscript text, therefore please incorporate the changes into your latest manuscript version. Please upload the final revised manuscript file with tracked changes.

- As a standard procedure, we edit the title and abstract of manuscripts to make them more accessible to a general readership. Please find the edited versions below my signature (175 words).

- Please add scale bars to the magnifications shown in Figure 5A and 7B.

We look forward to seeing a final version of your manuscript as soon as possible.

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Title and Abstract:

Retinoblastoma protein promotes uterine epithelial cell cycle arrest and necroptosis for embryo invasion

Retinoblastoma protein (RB) encoded by Rb1 is a prominent inducer of cell cycle arrest (CCA). The hormone progesterone (P4) promotes CCA in the uterine epithelium and previous studies indicated that P4 activates RB by reducing the phosphorylated, inactive form of RB . Here we show that embryo implantation is impaired in uterine-specific Rb1 knockout mice. We observe persistent cell proliferation of the Rb1-deficient uterine epithelium until embryo attachment, loss of epithelial necroptosis and trophoblast phagocytosis, which correlates with subsequent embryo invasion failure, indicating that Rb1-induced CCA and necroptosis of uterine epithelium are involved in embryo invasion. Pre-implantation P4 supplementation is sufficient to restore these defects and embryo invasion. In Rb1-deficient uterine epithelial cells, TNFα-primed necroptosis is impaired, which is rescued by the treatment with a CCA inducer thymidine or P4 through the upregulation of TNF receptor type 2. TNFα is expressed in the luminal epithelium and the embryo at the embryo attachment site. These results provide evidence that uterine Rb1-induced CCA is involved in

TNFα-primed epithelial necroptosis at the implantation site for successful embryo invasion.

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Referee #2:

Well revised. Thanks.

The authors have addressed all minor editorial requests.

2nd Revision - Editorial Decision 3rd Dec 2020

Dr. Yasushi Hirota
The University of Tokyo
Department of Obstetrics and Gynecology, Graduate School of Medicine
7-3-1 Hongo
Bunkyo-ku, Tokyo 113-8655
Japan

Dear Dr. Hirota,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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THINGS TO DO NOW:

You will receive proofs by e-mail approximately 2-3 weeks after all relevant files have been sent to our Production Office; you should return your corrections within 2 days of receiving the proofs.

Please inform us if there is likely to be any difficulty in reaching you at the above address at that time. Failure to meet our deadlines may result in a delay of publication, or publication without your corrections.

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EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Yasushi Hirota

Journal Submitted to: EMBO Reports

Manuscript Number: EMBOR-2020-50927V1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor
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http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We decided the sample sizes by taking into consideration proper power detecting differences, feasibility and the use of a minimal number of animals for the experiments.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	No statistical test was performed to estimate sample size for aminal studies.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No inclusion/exclusion criteria were used in our analyses.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No.
For animal studies, include a statement about randomization even if no randomization was used.	N/A
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No.
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	Yes, we specified the statistical analysis applied in the legend of every figure.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	N/A
Is there an estimate of variation within each group of data?	N/A

Is the variance similar between the groups that are being statistically compared?	N/A

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	We provided the information of antibodies in the Materials & Methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	We described the sources of primary cells and a cell line in the Materials & Methods section.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	We described the information of mice and their housing conditions in the Materials & Methods section.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal experiments were performed in accordance with the Animal Experiment Committee of the University of Tokyo.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Yes.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Experimental procedures were approved by the institutional review board of the University of Tokyo.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Yes, the signed informed consent for the use of tissues was obtained from each patient and the experiments were performed according to the principles in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	RNA-Seq datasets produced in this study are available in Sequence Read Archive.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A