F&S Science

Refining endometrial assembloids: a novel approach to 3D culture of the endometrium

--Manuscript Draft--

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Keywords:	Assembloid; endometrium; organoid; protocol optimization; in vitro model; implantation
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Abstract:	Enhancing the efficacy of three-dimensional endometrial assembloid models is pivotal for deepening our comprehension of endometrial physiology. We investigated the optimal ratio of organoids to stromal cells in these models, utilizing human endometrial biopsies. Morphological and viability analyses revealed that the 1:1 and 1:2 ratios were optimal until day 4, after which viability declined significantly by day 10, coinciding with a notable disappearance of stromal cells. To overcome this challenge, we developed a hanging-drop culture model, which enhanced cell survival and better replicated natural cellular organization. These findings underscore the significance of considering stromal cell dynamics in refining assembloid models for studying endometrial physiology and endometrial-related pathologies.
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	Felice Petraglia University of Florence felice.petraglia@unifi.it
	ayman Al-Hendy The University of Chicago aalhendy@BSD.Uchicago.edu

Opposed Reviewers:	
Response to Reviewers:	We would like to thank the reviewers for their valuable advice. We have carefully considered all issues mentioned in the reviewers' comments (see responses below). Answers to the reviewers' comments Reviewer 1: The Authors of manuscript number XFSS-D-24-00045, titled "Refining endometrial assembloids: a novel approach to 3D culture of the endometrium" have efficiently tested different parameters of a three-dimensional endometrial assembloid model and they are suggesting a change in the conventional method, in order to optimize this model. They present their preliminary data of the new model that is still under investigation. Good quality cell culture models to study endometrial function are needed and will aid the search for mechanisms involved in endometrial differentiation/decidualization and embryo implantation. The results of these short communication are interesting and as expected stromal cells gradually disappeared from Matrigel droplets and adhered to make their monolayer onto the plastic (as they usually do in vitro). The new model, with the Matrigel drop that hangs upside down, suggested by the authors, seems promising in order to overcome the stroma cell loss and is worth to be further investigated. Corrections: I would suggest the authors to rearrange the different parts of the manuscript in order to be clearer. In the study design they already include a description of the methods. In the results section, except the results in table 1, to include all the remaining parts of the figure 1 and their description: 1C and H are not mentioned and 1D-F are in the conclusion. Finally, in the conclusion discuss the findings and future experiments for the new very interesting approach of the upside-down drop. In addition, in figure 1 the different sections are not numbered correctly, there are two Fs and no H. We thank the reviewer for their encouraging comments and valuable advice. We have modified the manuscript and highlighted the changes. Here are the applied corrections: 1) I would suggest the authors to rearrange the different parts of the manuscript in order to be clearer. We thank the reviewer for this insight. We have rearranged the paragraphs and divided the sections more customarily into 'Introduction', 'Material and methods', 'Results', 'Discussion' and 'Conclusion' to improve clarity. 2) In the study design they already include a description of the methods. This issue has now been resolved; the title of this paragraph has been changed to 'Material and methods'. 3) In the results section, except the results in table 1, to include all the remaining parts of the figure 1 and their description: 1C and H are not mentioned and 1D-F are in the conclusion. We thank the reviewer for this remark. We have rearranged different parts of Figure 1 by splitting it into two and changing the order. All (sub-)figures are now correctly referred to in the appropriate places in the text: Figure 1A and B in material and methods (line 51), Figure 2A, B, C in results (lines 60, 61, 71) and Figure 2D, E, F in the discussion (lines 91, 94, 98). 4) Finally, in the conclusion discuss the findings and future experiments for the new very interesting approach of the upside-down drop. We appreciate this enthusiastic encouragement. We have added a paragraph to the conclusion to discuss our findings and outline experiments for this innovative approach (lines 109-113). 5) In addition, in figure 1 the different sections are not numbered correctly, there are two Fs and no H. Thank you for pointing this out. The previous issue of having two Fs and no H has been resolved and each sub-figure is now correctly labeled and referenced in the text (see above in section 3).

	Reviewer 2: Good job deserve future studies We are greatly encouraged by this comment and will press ahead with our investigations, thank you.
Additional Information:	
Question	**Response**

IREC Institut de Recherche Expérimentale et Clinique
Université catholique de Louvain
Secteur des Sciences de la Santé

Prof. M.M. Dolmans

Pôle de Gynécologie

UCL Université catholique de Louvain

Brussels, July 24, 2024
William Catherino, MD, PhD
Editor-in-Chief
Fertility and Sterility Science

Dear Editor-in-Chief,

Please find herewith our revised manuscript entitled "Refining endometrial assembloids: a novel approach to 3D culture of the endometrium" by Beaussart C, Rossi M, Stratopoulou CA, Zipponi M, Cacciottola L, Donnez J and Dolmans MM.

We would like to thank the reviewers for their valuable advice. We have carefully considered all issues mentioned in the reviewers' comments (see responses below).

We believe these changes enhance the clarity and comprehensiveness of our manuscript and hope it now meets the high standards of Fertility and Sterility Science. Thank you for your consideration of this manuscript.

Sincerely,

Prof. M.M. DOLMANS

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TITLE: Refining endometrial assembloids: a novel approach to 3D culture of the endometrium

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KEY WORDS: Assembloid; endometrium; organoid; protocol optimization; in vitro model; implantation.

INTRODUCTION

Human endometrium is a complex structure that plays a key role in embryo implantation. Successful implantation is crucial to progression of pregnancy, so elucidating the mechanisms behind this process could be fundamental to boosting its chances of success. However, because of the limited window of implantation (WOI) and challenges associated with accessing the uterus in vivo during this short span, including ethical considerations, our understanding of this phenomenon remains incomplete. For this reason, researchers often resort to in vitro models to investigate implantation (1,2). Reports have recently emerged on novel three-dimensional endometrial assembloids, which consist of gland-like epithelial organoids seeded with primary stromal cells, providing a physiologically relevant platform to scrutinize implantation dynamics (3,4,5). While promising, this new model still requires refinement for optimal efficacy. Our goal was to determine the most suitable ratio of organoids to stromal cells within assembloids and assess their viability over time, looking to optimize their use in further studies.

MATERIAL AND METHODS

Use of human biopsies (n=3) for this study was approved by the Ethics Committee of Cliniques Universitaires Saint-Luc (CUSL) and the Université Catholique de Louvain on August 31, 2020, and amended on July 23, 2023 (reference 2020/14AOU/410). Endometrial biopsies were collected straight after hysterectomy and transported on ice to be processed in the laboratory. Specimens were cut into 1 mm^3 pieces, digested with collagenase and filtered through 100 μm^2 filters to dissociate epithelial and stromal fractions. Stromal cells were cultured in Dulbecco's modified Eagle medium (DMEM) and Ham's F-12 nutrient mixture (1:1), enriched with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in T25 cell culture flasks. Epithelial glands were embedded in Matrigel domes on 48-well plates and cultured in expansion medium, as described by Turco et al. (1). Both cultures were passaged every 7-10 days and medium was changed every 2-3 days.

Forty assembloids per biopsy were generated by carefully combining glandular organoids and stroma together using different ratios of stromal cells relative to glands (2:1, 1:1, 1:2, 1:4). The 1:1 ratio corresponded to one T25 flask of 100% confluent stromal cells merged with 5 wells of organoids in order to obtain 5 wells of assembloids. Within these assembloids, immunohistochemistry detected epithelial marker CK22 (Figure 1A) and stromal marker CD10 (Figure 1B), confirming their epithelial and stromal origin. The assembloids were kept in culture in organoid expansion medium supplemented with 2% FBS and analyzed at 3 different timepoints: day 0 (assembloid creation), day 4 and day 10. The quality of assembloid cultures was assessed in terms of: (i) morphology; (ii) viability by calcein AM/ethidium homodimer assays; and (iii) proliferation and apoptosis status by Ki67 and caspase-3 immunohistochemistry respectively.

RESULTS

Morphology showed that glands and stromal cells were able to maintain their structural integrity and spatial arrangement within Matrigel over the first few days of culture, namely day 0 to 4 (Figure 2A and B). Stromal cells are connective cells that provide tissue with structural support. Their arrangement is therefore crucial to delivering structural integrity to glands, as well as transport of nutrients and waste products, and effective cell-cell communication. This well structured organization was not, however,

observed with the 2:1 ratio, where Matrigel domes detached from the wells after two days of culture due to excessive numbers of stromal cells.

Morphology also established that stromal cells gradually disappear from Matrigel droplets, either through cell death or cell migration to the bottom of the well. Indeed, by day 10, the majority of stromal cells appeared to have organized themselves into their natural monolayer architecture, attaching to the plastic support and no longer serving the purpose of the model.

Viability tests determined relative viability compared to day 0 at 45%, 91%, 82% and 94% for ratios 2:1, 1:1, 1:2 and 1:4 respectively, but this had fallen dramatically by day 10 to just 24%, 35%, 51% and 47% (** p value=0.0022) (Table 1) (Figure 2C).

Proliferation assessment showed no variation across the 1:1, 1:2 and 1:4 ratios, indicating consistent cell proliferation rates across these ratios (Table 1). However, significant apoptosis was observed on day 10 for ratio 1:1 [**p value(glands)=0.0099; **p value(stromal cells)=0.0017], and 2:1 [***p value(glands)=0.0003; *p value (stromal cells)=0.0152], also showing an increased apoptosis rate with the 1:4 ratio on day 0 [***p value(glands)=0.0007; **p value (stromal cells)=0.0065] and on day 4 [*p value(glands)= 0,0257; **p value(stromal cells)=0.0013] (Table 1). T-tests to compare groups were performed using Graphpad Prism 8.0.1 software. Ap

DISCUSSION

The 2:1 ratio was quickly found to be inadequate in terms of morphology due to detachment of the Matrigel dome and markedly decreased viability, leading us to exclude it as an appropriate model. Apoptosis assessment demonstrated that the 1:4 ratio may not be conducive to cell survival either, ruling it out too. We may therefore conclude that the 1:1 and 1:2 ratios are the best options for optimal organization and survival of co-culture. With both ratios, structural integrity, viability and apoptosis were optimally and similarly maintained from day 0 until day 4, giving us a favorable timeframe for use.

Morphology and apoptosis observations on day 10 suggest that disappearance of stromal cells with subsequent death and disorganization could be due to the model itself, so we are now developing a new culture model. In this model, the Matrigel drop hangs upside down, using gravity to prevent progressive disappearance of stromal cells (Figure 2D). Our preliminary results are promising, addressing issues previously encountered with the conventional culture technique. Indeed, morphology analysis of hanging-drop assembloid cultures indicates that stromal cells survive in Matrigel drops, even up to day 10. This has also been confirmed by hematoxylin and eosin staining (Figure 2E). By maintaining cells in structured co-culture, this method likely better mimics the natural organization and interaction between different endometrial cell types, offering a more accurate and robust culture approach. Viability assessment also revealed enhanced viability on day 10 compared to conventional culture, allowing cells to survive and thrive for longer, extending the previous 4-day timeframe (Figure 2F). All this yields greater flexibility in designing and conducting research experiments.

CONCLUSION

In conclusion, the 1:1 and 1:2 ratios of stromal cells relative to glands were found to be best suited to maintaining structural integrity, viability and the apoptosis balance of co-cultures from day 0 to day 4. However, the significant decrease in viability and disappearance of stromal cells by day 10 in conventional culture point to the need for a more sophisticated model. Development of the hanging-drop assembloid

model appears to be a promising solution, as it addresses these issues and allows for prolonged culture with better-maintained cell structure and viability.

Future experiments will focus on further refining the hanging-drop model to enhance its reliability and robustness. This includes evaluating the functional aspects of endometrial assembloids, such as hormone responsiveness and gene expression profiles. These improvements aim to establish a more physiologically relevant and long-lasting in vitro model for studying implantation dynamics and other aspects of endometrial biology.

CONFLICT OF INTEREST DISCOSURE (COI): CB, MR, MZ, CAS, LC, JD and MMD do not have any competing interests to declare.

ACKNOWLEDGEMENTS: This study was supported by grants from the Fondation Saint-Luc awarded to CB and MMD, and funds from the Fonds National de la Recherche Scientifique de Belgique (FNRS) (FC 52391 awarded to MZ, FNRS FC39041 awarded to CAS and FNRS 5/4/150/5 awarded to MMD).
This research has been conducted using specimens from the CUSL Biobank collection (Belgian accreditation BB190044).
The authors thank Mira Hryniuk, BA, for reviewing the English language of the manuscript, Deborah Godefroidt for her administrative assistance.

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4. Gnecco JS, Brown A, Buttrey K, Ives C, Goods BA, Baugh L et al. Organoid co-culture model of the human endometrium in a fully synthetic extracellular matrix enables the study of epithelial-stromal crosstalk. Med 2023;4(8):554–579.e9.
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LEGENDS TO FIGURES

Figure 1. Endometrial assembloids A. Immunohistochemistry: epithelial marker CK22. B. Immunohistochemistry: stromal marker CD10.

Figure 2. Endometrial assembloids A. Conventional assembloid culture method: organoids and stromal cells suspended in a Matrigel dome, seeded inside a well and supplemented with culture medium. B. H&E

staining of a conventional assembloid culture on day 10 at ratio 1:1 (x10). C. Viability assessment of a conventional assembloid culture on day 10 at ratio 1:1. D. Hanging-drop assembloid culture method: organoids and stromal cells suspended in a hanging Matrigel droplet, seeded on an insert inside a well filled with culture medium. E. H&E staining of a hanging-drop assembloid culture derived from the same biopsy on day 10 at ratio 1:1 (x10). F. Viability assessment of a hanging-drop assembloid culture derived from the same biopsy on day 10 at ratio 1:1.

TABLES AND FIGURES

Table 1. Viability, proliferation and apoptosis results. Viability expressed in % compared to day 0, and proliferation and apoptosis expressed in % of stained cells out of the total number of cells.

	Day 0			Day 4			Day 10		
	Viability	Proliferation	Apoptosis	Viability	Proliferation	Apoptosis	Viability	Proliferation	Apoptosis
2:1	100%	N.A.	N.A.	45%	N.A.	N.A.	24%	N.A.	N.A.
1:1	100%	Glands 28% Stroma 50%	Glands 4% Stroma 2%	91%	Glands 20% Stroma 40%	Glands 3% Stroma 4%	35%	Glands 22% Stroma 33%	Glands 15% Stroma 13%
1:2	100%	Glands 26% Stroma 24%	Glands 2% Stroma 4%	82%	Glands 30% Stroma 16%	Glands 3% Stroma 11%	51%	Glands 21% Stroma 16%	Glands 10% Stroma 16%
1:4	100%	Glands 32% Stroma 20%	Glands 13% Stroma 12%	94%	Glands 16% Stroma 20%	Glands 14% Stroma 14%	47%	Glands 20% Stroma 19%	Glands 15% Stroma 17%

Figure 1.

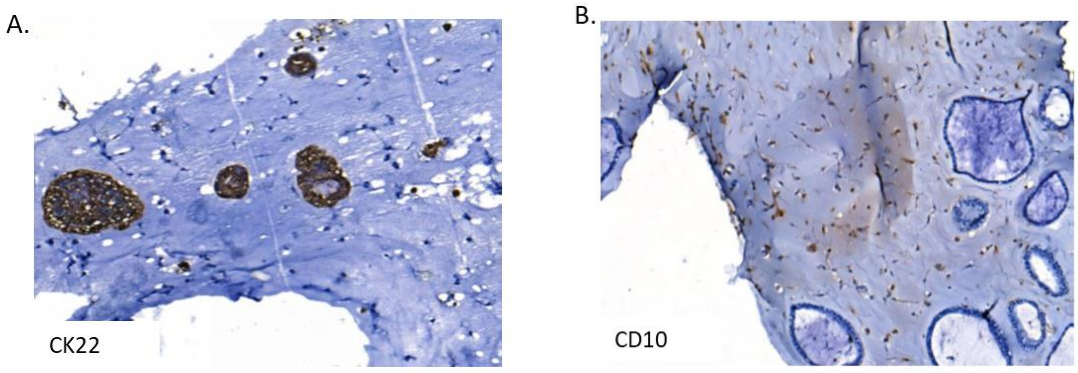
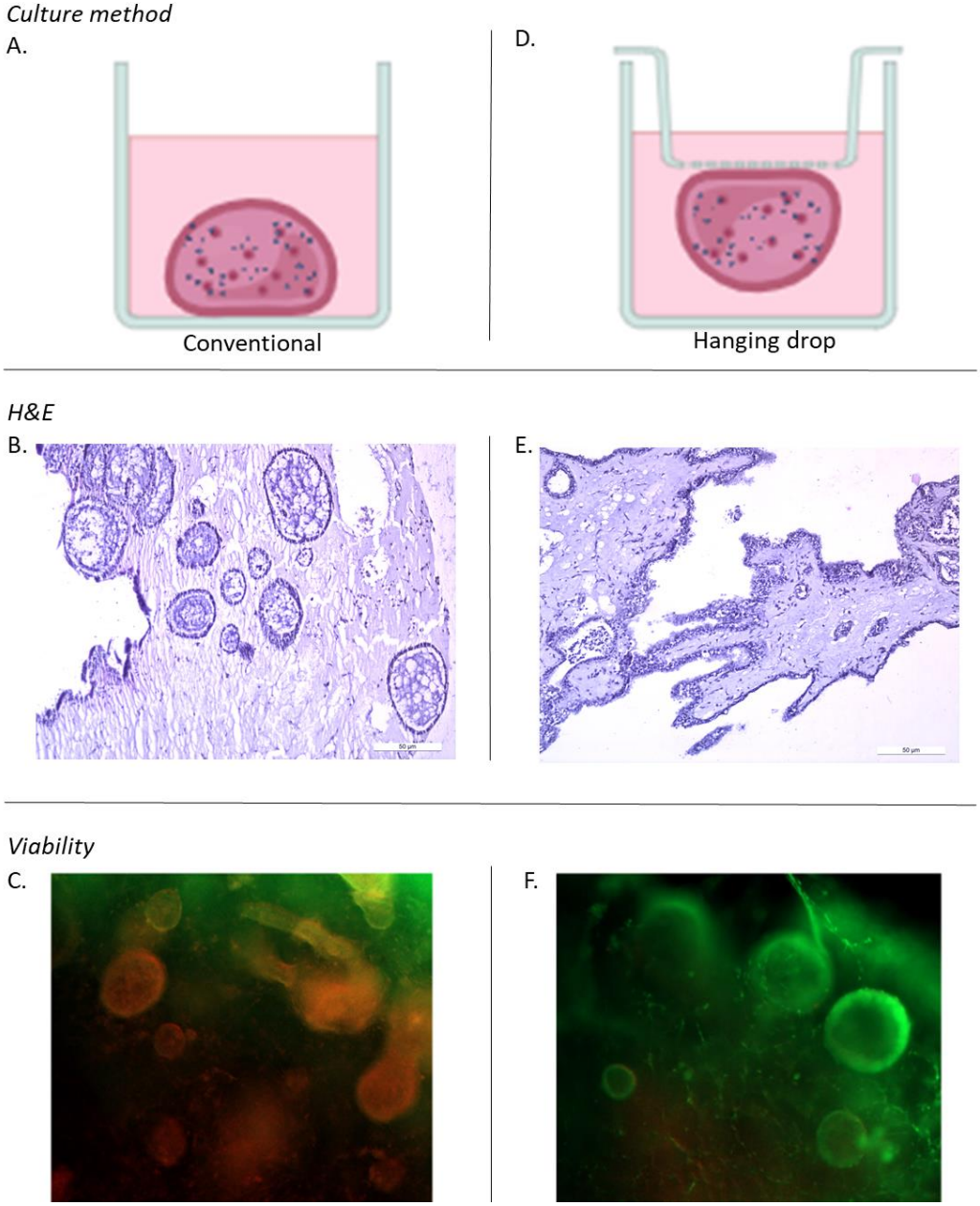


Figure 2.


Culture method
A.
D.
Conventional
Hanging drop
H&E
B.
E.
Viability
C.
F.

ICMJE DISCLOSURE FORM

Date: 5/6/2024

Your Name: Beaussart Chloé, Rossi Margherita, Zipponi Margherita, Stratopoulou Christina Anna, Cacciottola Luciana, Donnez Jacques and Dolmans Marie-Madeleine

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Fertility and Sterility.

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Manuscript title: Refining endometrial assembloids : a novel approach to 3D culture of the endometrium

Corresponding author: Marie - Madeleine Dolmans

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KEY WORDS: Assembloid; endometrium; organoid; protocol optimization; in vitro model; implantation.

INTRODUCTION

Human endometrium is a complex structure that plays a key role in embryo implantation. Successful implantation is crucial to progression of pregnancy, so elucidating the mechanisms behind this process could be fundamental to boosting its chances of success. However, because of the limited window of implantation (WOI) and challenges associated with accessing the uterus in vivo during this short span, including ethical considerations, our understanding of this phenomenon remains incomplete. For this reason, researchers often resort to in vitro models to investigate implantation (1,2). Reports have recently emerged on novel three-dimensional endometrial assembloids, which consist of gland-like epithelial organoids seeded with primary stromal cells, providing a physiologically relevant platform to scrutinize implantation dynamics (3,4,5). While promising, this new model still requires refinement for optimal efficacy. Our goal was to determine the most suitable ratio of organoids to stromal cells within assembloids and assess their viability over time, looking to optimize their use in further studies.

MATERIAL AND METHODS

Use of human biopsies (n=3) for this study was approved by the Ethics Committee of Cliniques Universitaires Saint-Luc (CUSL) and the Université Catholique de Louvain on August 31, 2020, and amended on July 23, 2023 (reference 2020/14AOU/410). Endometrial biopsies were collected straight after hysterectomy and transported on ice to be processed in the laboratory. Specimens were cut into 1 mm^3 pieces, digested with collagenase and filtered through 100 μm^2 filters to dissociate epithelial and stromal fractions. Stromal cells were cultured in Dulbecco's modified Eagle medium (DMEM) and Ham's F-12 nutrient mixture (1:1), enriched with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in T25 cell culture flasks. Epithelial glands were embedded in Matrigel domes on 48-well plates and cultured in expansion medium, as described by Turco et al. (1). Both cultures were passaged every 7-10 days and medium was changed every 2-3 days.

Forty assembloids per biopsy were generated by carefully combining glandular organoids and stroma together using different ratios of stromal cells relative to glands (2:1, 1:1, 1:2, 1:4). The 1:1 ratio corresponded to one T25 flask of 100% confluent stromal cells merged with 5 wells of organoids in order to obtain 5 wells of assembloids. Within these assembloids, immunohistochemistry detected epithelial marker CK22 (Figure 1A) and stromal marker CD10 (Figure 1B), confirming their epithelial and stromal origin. The assembloids were kept in culture in organoid expansion medium supplemented with 2% FBS and analyzed at 3 different timepoints: day 0 (assembloid creation), day 4 and day 10. The quality of assembloid cultures was assessed in terms of: (i) morphology; (ii) viability by calcein AM/ethidium homodimer assays; and (iii) proliferation and apoptosis status by Ki67 and caspase-3 immunohistochemistry respectively.

RESULTS

Morphology showed that glands and stromal cells were able to maintain their structural integrity and spatial arrangement within Matrigel over the first few days of culture, namely day 0 to 4 (Figure 2A and B). Stromal cells are connective cells that provide tissue with structural support. Their arrangement is therefore crucial to delivering structural integrity to glands, as well as transport of nutrients and waste products, and effective cell-cell communication. This well structured organization was not, however,

observed with the 2:1 ratio, where Matrigel domes detached from the wells after two days of culture due to excessive numbers of stromal cells.

Morphology also established that stromal cells gradually disappear from Matrigel droplets, either through cell death or cell migration to the bottom of the well. Indeed, by day 10, the majority of stromal cells appeared to have organized themselves into their natural monolayer architecture, attaching to the plastic support and no longer serving the purpose of the model.

Viability tests determined relative viability compared to day 0 at 45%, 91%, 82% and 94% for ratios 2:1, 1:1, 1:2 and 1:4 respectively, but this had fallen dramatically by day 10 to just 24%, 35%, 51% and 47% (** p value=0.0022) (Table 1) (Figure 2C).

Proliferation assessment showed no variation across the 1:1, 1:2 and 1:4 ratios, indicating consistent cell proliferation rates across these ratios (Table 1). However, significant apoptosis was observed on day 10 for ratio 1:1 [**p value(glands)=0.0099; **p value(stromal cells)=0.0017], and 2:1 [***p value(glands)=0.0003; *p value (stromal cells)=0.0152], also showing an increased apoptosis rate with the 1:4 ratio on day 0 [***p value(glands)=0.0007; **p value (stromal cells)=0.0065] and on day 4 [*p value(glands)= 0,0257; **p value(stromal cells)=0.0013] (Table 1). T-tests to compare groups were performed using Graphpad Prism 8.0.1 software. Ap

DISCUSSION

The 2:1 ratio was quickly found to be inadequate in terms of morphology due to detachment of the Matrigel dome and markedly decreased viability, leading us to exclude it as an appropriate model. Apoptosis assessment demonstrated that the 1:4 ratio may not be conducive to cell survival either, ruling it out too. We may therefore conclude that the 1:1 and 1:2 ratios are the best options for optimal organization and survival of co-culture. With both ratios, structural integrity, viability and apoptosis were optimally and similarly maintained from day 0 until day 4, giving us a favorable timeframe for use.

Morphology and apoptosis observations on day 10 suggest that disappearance of stromal cells with subsequent death and disorganization could be due to the model itself, so we are now developing a new culture model. In this model, the Matrigel drop hangs upside down, using gravity to prevent progressive disappearance of stromal cells (Figure 2D). Our preliminary results are promising, addressing issues previously encountered with the conventional culture technique. Indeed, morphology analysis of hanging-drop assembloid cultures indicates that stromal cells survive in Matrigel drops, even up to day 10. This has also been confirmed by hematoxylin and eosin staining (Figure 2E). By maintaining cells in structured co-culture, this method likely better mimics the natural organization and interaction between different endometrial cell types, offering a more accurate and robust culture approach. Viability assessment also revealed enhanced viability on day 10 compared to conventional culture, allowing cells to survive and thrive for longer, extending the previous 4-day timeframe (Figure 2F). All this yields greater flexibility in designing and conducting research experiments.

CONCLUSION

In conclusion, the 1:1 and 1:2 ratios of stromal cells relative to glands were found to be best suited to maintaining structural integrity, viability and the apoptosis balance of co-cultures from day 0 to day 4. However, the significant decrease in viability and disappearance of stromal cells by day 10 in conventional culture point to the need for a more sophisticated model. Development of the hanging-drop assembloid

model appears to be a promising solution, as it addresses these issues and allows for prolonged culture with better-maintained cell structure and viability.

Future experiments will focus on further refining the hanging-drop model to enhance its reliability and robustness. This includes evaluating the functional aspects of endometrial assembloids, such as hormone responsiveness and gene expression profiles. These improvements aim to establish a more physiologically relevant and long-lasting in vitro model for studying implantation dynamics and other aspects of endometrial biology.

CONFLICT OF INTEREST DISCOSURE (COI): CB, MR, MZ, CAS, LC, JD and MMD do not have any competing interests to declare.

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LEGENDS TO FIGURES

Figure 1. Endometrial assembloids A. Immunohistochemistry: epithelial marker CK22. B. Immunohistochemistry: stromal marker CD10.

Figure 2. Endometrial assembloids A. Conventional assembloid culture method: organoids and stromal cells suspended in a Matrigel dome, seeded inside a well and supplemented with culture medium. B. H&E

staining of a conventional assembloid culture on day 10 at ratio 1:1 (x10). C. Viability assessment of a conventional assembloid culture on day 10 at ratio 1:1. D. Hanging-drop assembloid culture method: organoids and stromal cells suspended in a hanging Matrigel droplet, seeded on an insert inside a well filled with culture medium. E. H&E staining of a hanging-drop assembloid culture derived from the same biopsy on day 10 at ratio 1:1 (x10). F. Viability assessment of a hanging-drop assembloid culture derived from the same biopsy on day 10 at ratio 1:1.

TABLES AND FIGURES

Table 1. Viability, proliferation and apoptosis results. Viability expressed in % compared to day 0, and proliferation and apoptosis expressed in % of stained cells out of the total number of cells.

	Day 0			Day 4			Day 10		
	Viability	Proliferation	Apoptosis	Viability	Proliferation	Apoptosis	Viability	Proliferation	Apoptosis
2:1	100%	N.A.	N.A.	45%	N.A.	N.A.	24%	N.A.	N.A.
1:1	100%	Glands 28% Stroma 50%	Glands 4% Stroma 2%	91%	Glands 20% Stroma 40%	Glands 3% Stroma 4%	35%	Glands 22% Stroma 33%	Glands 15% Stroma 13%
1:2	100%	Glands 26% Stroma 24%	Glands 2% Stroma 4%	82%	Glands 30% Stroma 16%	Glands 3% Stroma 11%	51%	Glands 21% Stroma 16%	Glands 10% Stroma 16%
1:4	100%	Glands 32% Stroma 20%	Glands 13% Stroma 12%	94%	Glands 16% Stroma 20%	Glands 14% Stroma 14%	47%	Glands 20% Stroma 19%	Glands 15% Stroma 17%

Figure 1.

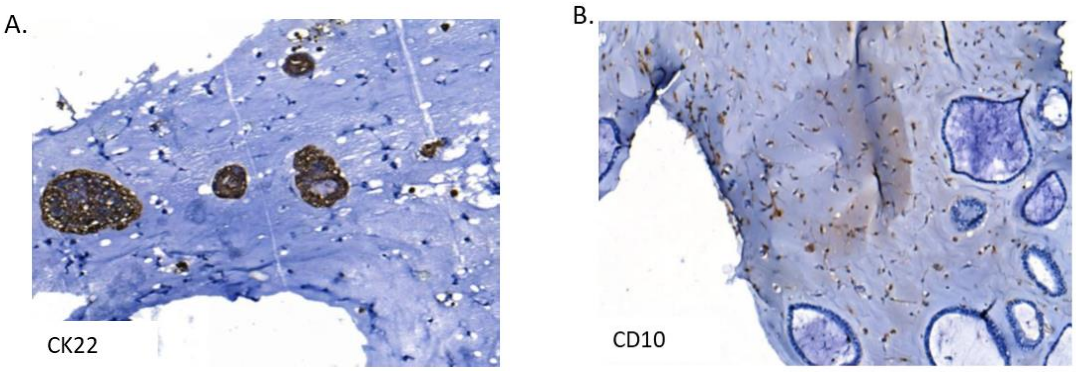
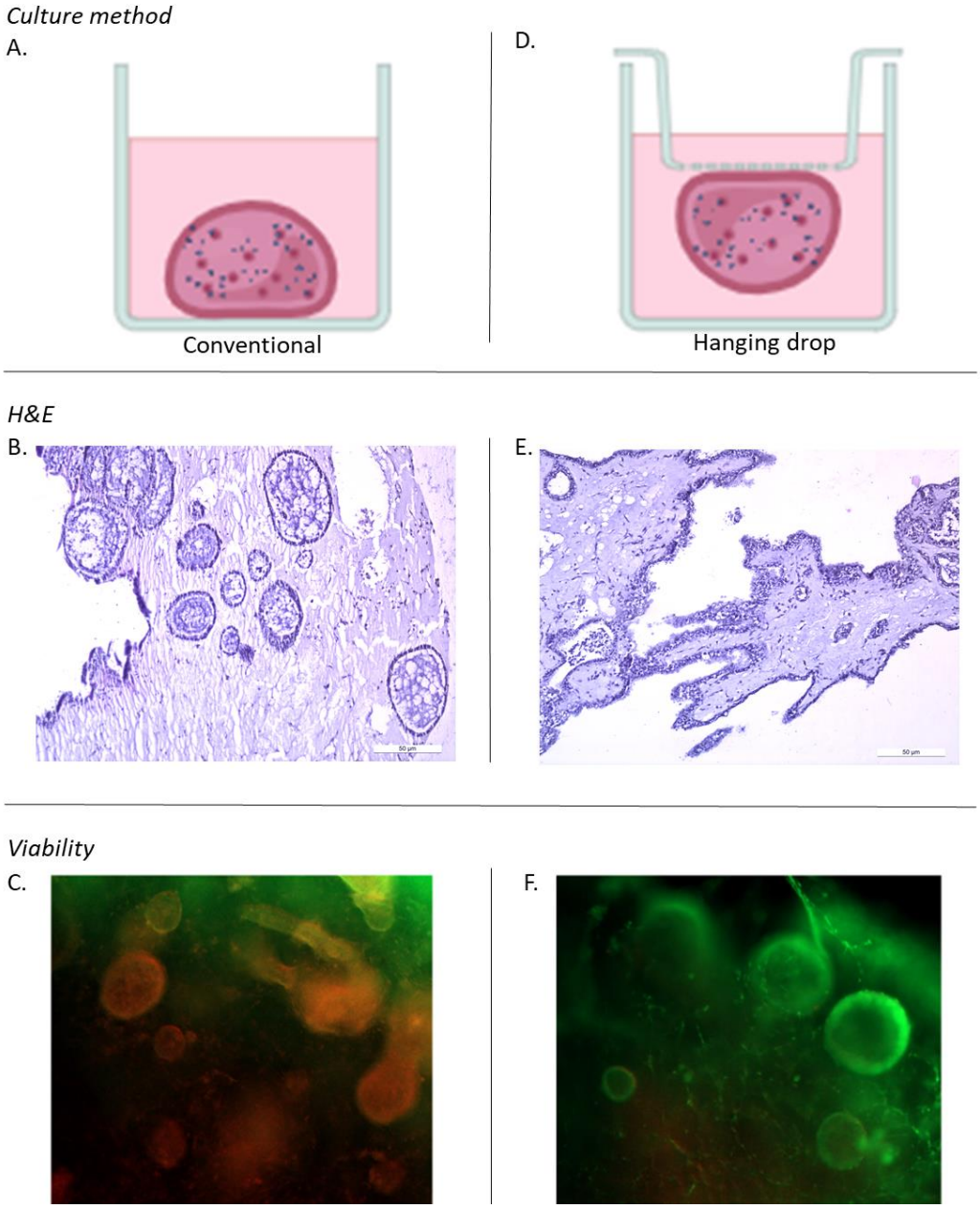


Figure 2.


Culture method
A.
D.
Conventional
Hanging drop
H&E
B.
E.
Viability
C.
F.