RBMO

ELSEVIER

ARTICLE

# Organoids can be established reliably from cryopreserved biopsy catheter-derived endometrial tissue of infertile women

## BIOGRAPHY

Bich Bui graduated from Maastricht University's Medical School in 2016, and in 2017 started her PhD in Reproductive Medicine combined with her job as a fertility physician at the University Medical Centre Utrecht. Her research focuses on the role of the endometrium in unexplained reproductive diseases.

Bich Ngoc Bui[1,*], Matteo Boretto[2], Hiroto Kobayashi[3], Marliek van Hoesel[1], Gaby S. Steba[1], Nienke van Hoogenhuijze[1], Frank J.M. Broekmans[1], Hugo Vankelecom[2], Helen L. Torrance[1]

## KEY MESSAGE

Organoids can be reliably established from cryopreserved endometrial tissue of infertile women and are similar to those derived from freshly biopsied tissue of the same patient. With the use of biobanked endometrial tissue, future organoid-based fertility research will no longer be limited to clinics situated near organoid laboratories.

## ABSTRACT

**Research question:** Can organoids be established from endometrial tissue of infertile women and does tissue cryopreservation allow for establishment of organoids comparable to organoids derived from freshly biopsied endometrial tissue?

**Design:** Endometrial tissue was obtained from six infertile women through minimally invasive biopsy using a Pipelle catheter and subjected to organoid development, immediately after biopsy as well as after tissue cryopreservation. Organoid formation efficiency, morphology, expandability potential, endometrial marker expression (immunostaining and reverse transcription quantitative real-time polymerase chain reaction) and hormonal responsiveness (after oestradiol and progesterone treatment) were assessed.

**Results:** Organoids established from both fresh and frozen tissue at comparable efficiency could be passaged long-term and showed similar morphology, i.e. cystic with a central lumen lined by a single epithelial cell layer. They also exhibited comparable expression of endometrial markers and proliferative activity (Ki67 expression). Finally, organoids from freshly biopsied and cryopreserved endometrial tissue showed similar responses to oestradiol and progesterone treatment.

**Conclusions:** Organoids can be established from cryopreserved endometrial tissue of infertile women and cryopreservation of the biopsy does not affect organoid formation and overall organoid characteristics. Cryopreservation of biopsies for later organoid development facilitates sample collection from any fertility clinic, not just the ones near an organoid laboratory.

[1] Department of Reproductive Medicine and Gynaecology, University Medical Centre Utrecht, Heidelberglaan 100, CX Utrecht 3584, the Netherlands
[2] Department of Development and Regeneration, Cluster of Stem Cell and Developmental Biology, Unit of Stem Cell Research, KU Leuven (University of Leuven), Herestraat 49, Leuven 3001, Belgium
[3] Department of Anatomy and Structural Science, Yamagata University Faculty of Medicine, 2-2-2 Iida-nishi, Yamagata 990-9585, Japan


*Corresponding author. E-mail address: b.n.bui@umcutrecht.nl (B N Bui). https://doi.org/10.1016/j.rbmo.2020.03.019 1472-6483/
Declaration: The authors report no financial or commercial conflicts of interest.

## KEYWORDS

Cryopreservation
Endometrial biopsy catheter
Endometrium
Infertility
Minimally invasive
Organoid

## INTRODUCTION

The endometrium undergoes a series of functional and morphological changes during the menstrual cycle that renders it temporarily receptive to embryo implantation (*Kodaman and Taylor, 2004*). During the proliferative phase, developing follicles produce increasing oestrogen levels, inducing proliferation of the endometrial epithelial and stromal cells and pseudostratification of the epithelium (*Noyes et al., 1975*). After ovulation, the corpus luteum is formed, which produces progesterone, marking the start of the secretory phase. This phase is characterized by cessation of endometrial proliferation, formation of epithelial basal vacuoles, increased secretion by luminal epithelial glands and stromal decidualization to prepare the endometrium for embryo implantation (*Noyes et al., 1975*).

Infertility is common, but the cause often remains unexplained (*van der Steeg et al., 2007*). Research into the role of the endometrium in infertility has long been hampered owing to limited tissue availability in laboratories and lack of access to clinical biopsies.

In the past decade, three-dimensional in-vitro structures called 'organoids' have been developed from diverse tissues (*Clevers, 2016*). Organoids self-organize from tissue sub-units or stem cells under specific culture conditions and recapitulate the original organ's key biological properties, thereby offering the possibility to mimic disease- and patient-specific phenotypes (*Clevers, 2016*; *Kretzschmar and Clevers, 2016*). As organoids are robustly expandable while remaining phenotypically and genetically stable (*Clevers, 2016*; *Kretzschmar and Clevers, 2016*), they make broad omics analysis, i.e. genomics, transcriptomics and proteomics, possible. Organoids may, therefore, provide a valuable tool to perform more in-depth studies into the role of the endometrium in various unexplained reproductive disease states.

Recently, organoids were developed from human endometrium sampled by laparoscopy (*Boretto et al., 2017*) and endometrial biopsy catheter (*Turco et al., 2017*). Organoid-based research is currently limited to clinics with organoid laboratories nearby because freshly obtained tissue is used. It is unknown whether endometrial tissue cryopreservation affects organoid development and characteristics. If organoid development from cryopreserved endometrial tissue is found reliable, it will allow protocols to be established using banked tissues (*Walsh et al., 2016*).

The aims of this study were, to investigate whether organoids can be established from cryopreserved tissue from infertile women and whether tissue cryopreservation allows for establishment of organoids comparable to organoids derived from freshly biopsied endometrial tissue.

## MATERIALS AND METHODS

### Endometrial organoid culture

Ethical approval was obtained from the Institutional Review Board (15-495, 30 November 2015 and 17-592, 28 September 2017) and the Biobank Research Ethics Committee (17-784, 22 January 2018) of the University Medical Centre Utrecht. Endometrial tissue was collected from 6 infertile patients using an endometrial biopsy catheter (Pipelle de Cornier, CCD International, France) (Supplementary Table 1). Endometrial tissue was collected in Dulbecco Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F-12) (Thermo Fisher Scientific, Waltham, MA, USA) with 5% fetal bovine serum (FBS) (Sigma-Aldrich, St Louis, Mo, USA) and 2% penicillin–streptomycin (10,000 U/ml) (Thermo Fisher Scientific, Waltham, MA, USA). Of each freshly isolated biopsy, one part was directly used for organoid development and one part was cryopreserved and used for later organoid culture. Before cryopreservation, tissue was washed with $Ca^{2+}$/$Mg^{2+}$-free phosphate buffered saline (PBS) (-), (Thermo Fisher Scientific, Waltham, MA, USA), cut into small pieces, collected in cryopreservation medium (DMEM/F-12 with 30% FBS and 10% dimethyl sulfoxide (DMSO) (Sigma Aldrich, St Louis, Mo, USA), slowly frozen at –80°C in a Mr Frosty Freezing Container (Thermo Fisher Scientific, Waltham, MA, USA) and stored in liquid nitrogen at –196°C until organoid development (Supplementary Table 1). On the day of organoid culture, the cryopreserved tissue part was rapidly thawed at 37°C and diluted with DMEM/F-12 with 10% FBS (1:10). After centrifugation (5 min, 200 g) and removal of the supernatant, tissue dissociation, organoid culturing and passaging were carried out as previously described (*Boretto et al., 2017*).

In brief, endometrial tissue was minced into small pieces, extensively washed in PBS (-) and incubated in 1 mg $ml^{-1}$ collagenase IV (Thermo Fisher Scientific, Waltham, MA, USA) with Y-27632 (5 µM) (Merck Millipore, Burlington, Massachusetts, USA) at 37°C for 1–2 h with gentle agitation. Tissue pieces were mechanically triturated every 20 min. The digestion was inactivated by 1:1 dilution with DMEM/F-12 with 10% FBS. Tissue fragments were further incubated with TrypLE (Thermo Fisher Scientific, Waltham, MA, USA) and Y-27632 (5 µM) for 10 min at 37°C. TrypLE was inactivated by dilution with DMEM/F-12 (1:20). After centrifugation (5 min, 200 g) the pellet of endometrial glandular fragments was resuspended in 70% Matrigel (Corning Inc, New York, USA) and 30% DMEM/F-12 with Y-27632 (10 µM) and incubated at 37°C and 5% $CO_2$ to allow the Matrigel to solidify, after which pre-warmed endometrial organoid culture medium (*Boretto et al., 2017*) was added and refreshed every 48 h. Outgrowing organoids were passaged every 10–14 days in a ratio of 1:4 to 1:5. Briefly, the organoid-containing Matrigel drops were dissolved in ice-cold DMEM/F-12, centrifugated, resuspended in TrypLE for 10 min at 37°C, inactivated by dilution with DMEM/F-12 (1:1), mechanically triturated, resuspended in 70% Matrigel and 30% DMEM/F-12 and cultured at 37°C and 5% $CO_2$ as described above. Unless otherwise stated, organoids from low passages (1 to 4) were used for all experiments.

### Organoid formation efficiency

After tissue dissociation as previously described (*Boretto et al., 2017*), cells were resuspended in Trypan Blue (1:1 dilution) and the number of viable and dead cells was counted using a haemocytometer (Blaubrand, Neubauer improved) (Sigma Aldrich, St Louis, Mo, USA). A total of 7500 viable cells per well (of a 48-wells plate) were resuspended in 70% Matrigel and 30% DMEM/F-12 and drops of 20 µl were seeded per well. Organoid formation efficiency after 7 and 10 days of culture was calculated as follows (number of organoids with diameter ≥100 µm/number of cells seeded) x 100%. A cut-off of 100 µm was chosen for counting purposes.

### Hormonal treatment of endometrial organoids

Hormonal treatment was started 96 h after passaging. Organoids were either treated with endometrial organoid culture medium (*Boretto et al., 2017*) supplemented with oestradiol (1 nM) only for 7 days or with progesterone (200 ng $ml^{-1}$) and oestradiol (0.1 nM) for 7 days after priming with oestradiol (1 nM) for 48 h, after which they were harvested for staining, transmission electron microscopy, or gene expression analysis.

### Histological and immunohistochemical analysis

Organoids were released from the Matrigel using ice-cold DMEM/F-12 and fixed in paraformaldehyde, 4% in PBS (-) for 1 h at room temperature. Organoids were washed three times in PBS (-) and stored in 70% ethanol at 4°C until they were paraffin-embedded and cut into 5-µm sections. Slides were deparaffinized at 58°C for 1 h and rehydrated in consecutive xylene and reverse ethanol gradients. Slides were then washed in purified water and PBS (-). Haematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) (Sigma Aldrich, St Louis, Mo, USA) stainings were carried out according to the manufacturer's instructions.

For immunohistochemical and immunofluorescence staining, antigen retrieval was executed in sodium citrate buffer (10 mM, pH 6) for 30 min at 95°C. Blocking was carried out at room temperature during 1 h with 10% donkey serum in blocking buffer (bovine serum albumin 2 mg $ml^{-1}$ and 0.15% glycine in PBS-Triton-X [PBS-T]). Sections were incubated overnight at 4°C with primary antibodies diluted in blocking buffer containing 1% donkey serum for the following antigens: acetylated α-tubulin, E-cadherin, oestrogen receptor α, ERα Ki67, progestagen-associated endometrial protein (PAEP) and progesterone receptor (PR) (Supplementary Table 2). As negative controls, isotype-specific immunoglobulins were used and primary antibodies were omitted to control for non-specific binding of secondary antibodies (Supplementary Figure). After incubation with the primary antibody, slides were washed three times in PBS-T.

For immunofluorescence staining, sections were incubated for 1 h at room temperature with Alexa Fluor 555 donkey anti-mouse (IgG) (A31570) (Life Technologies, Carlsbad, CA, USA) or Alexa Fluor 488 donkey anti-rabbit (IgG) (A21206) (Thermo Fisher Scientific, Thermo Fisher Scientific, Waltham, MA, USA) diluted (1:1000) in blocking buffer with 1% donkey serum. After two washes with PBS-T, sections were mounted with Vectashield Mounting Medium containing DAPI (Vector Laboratories, Burlingame, CA, USA).

For immunohistochemical staining, sections were incubated with 0.3% H2O2 (Sigma-Aldrich, St Louis, Mo, USA) for 20 min at room temperature followed by 30 min incubation with secondary antibodies (ImmPRESS Universal anti-mouse/rabbit IgG) at room temperature. Staining was developed by applying diaminobenzidine (DAB) substrate-chromogen (Dako, Carpinteria, CA, USA) and haematoxylin as counterstain. Sections were dehydrated in ethanol 95% and 100% and xylene and mounted with DPX mounting medium (Sigma-Aldrich, St Louis, Mo, USA).

Imaging of the immunohistochemical, H&E and PAS stainings was carried out using a Leica DM5500 microscope, and the Axio Imager Z1 was used to record immunofluorescence stainings. Images were sharpened using the Unsharp Mask filter in Adobe Photoshop CC 2019 (amount 200%, radius 2.0 pixels, threshold 0 levels).

### Transmission electron microscopy

Transmission electron microscopy was carried out as previously described (*Boretto et al., 2017*). Organoids from passage three were fixed in 2.5% glutaraldehyde in Na-cacodylate buffer (0.1 M, pH 7.2–7.4) overnight at 4°C. Subsequently, organoids were resuspended in 2.0% low melting point agarose solution. Organoid-containing agarose blocks were incubated in 1.5% potassium ferrocyanide in Na-cacodylate buffer (0.1 M) mixed with 1.0% osmium tetroxide for 1 h on ice. After several washes with Na-cacodylate buffer, samples were placed in 0.1% tannic acid for 20 min at room temperature and subsequently incubated in 1.0% osmium tetroxide for 1 h at room temperature. Samples were washed with Na-cacodylate buffer again, followed by treatment with 1.0% uranyl acetate overnight at 4°C. Subsequently, samples were incubated in Walton's lead aspartate for 30 min at 60°C and dehydrated in graded ethanol series. Incubation in propylene oxide was carried out, followed by incubation in a mixture of epoxy resin and propylene oxide, which was then replaced by 100% epoxy resin and polymerized at 60°C for at least 2 days. Ultra-thin sections (70 nm) were cut with an Ultracut-T (UCT) ultramicrotome (Leica Microsystems, Milton Keynes, UK) and analysed using the JEM1400 (JEOL, Zaventem, Belgium) equipped with SIS Quemesa 11 Mpxl camera (Olympus Corporation, Shinjuku, Japan).

### Gene expression analysis

Gene expression analysis (*GAPDH*, as reference gene; *ESR1*, *FOXA2*, *LIF*, *MUC1*, *PAEP* and *PR*) was conducted as previously described (*Boretto et al., 2017*). See Supplementary Table 3 for primer sequences.

Total RNA was extracted from endometrial organoids using the RNeasy Micro Kit (Qiagen) according to manufacturer's instructions. The quality and concentration of RNA was assessed using NanoDrop Spectrophotometer ND-1000. For cDNA synthesis, total RNA was reverse transcribed using SuperScript III First-Strand Synthesis SuperMix (Thermo Fisher Scientific, Waltham, MA, USA). StepOnePlus Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) was used for reverse transcription quantitative real-time polymerase chain reaction analysis using Platinum SYBR Green qPCR SuperMix-UDG (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. Relative gene expression levels were calculated as $2^{-\Delta Ct}$, in which ΔCt = Ct(target gene) - Ct(reference gene).

### Statistical analysis

GraphPad Prism (version 7.04) was used for statistical analysis. Results were expressed in median (interquartile range), and the two-tailed Wilcoxon signed-rank test was used for comparison of data of endometrial organoids developed from freshly biopsied and cryopreserved tissue of the same patient. $P < 0.05$ was considered to indicate a statistically significant difference. Experiments were carried out with at least three biological replicates and analysed in at least two technical replicates, unless otherwise stated.

## RESULTS

Organoid formation efficiency was not found to be significantly different

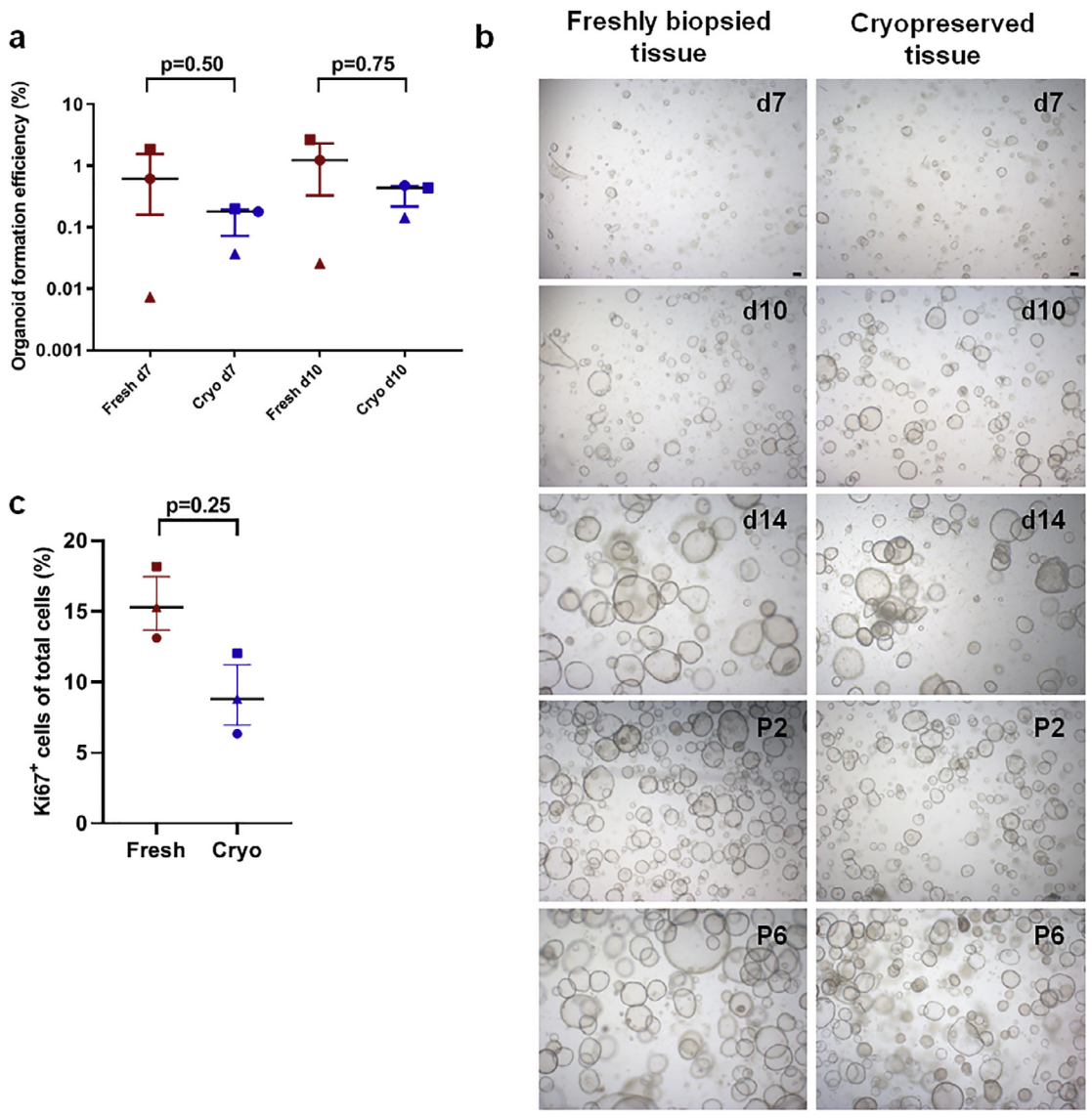


**FIGURE 1** Development and expansion of organoids from freshly obtained and cryopreserved endometrial tissue of infertile women. (a) Organoid formation efficiency (number of organoids with a diameter ≥100 µm/number of seeded cells x 100%) as determined 7 and 10 days after seeding of freshly biopsied and cryopreserved endometrial tissue fragments. The median organoid formation efficiency is shown on a $\log_{10}$ scale. Error bars display interquartile range. The following symbols correspond to patients 1, 2 and 3, respectively: bullet, square, triangle; (b) Representative bright-field pictures of organoids developing from freshly biopsied and cryopreserved endometrial tissue at day 7 (d7), d10 and d14 after seeding, at passage 2 (P2) and at P6. Scale bars 100 µm; (c) Proportion of proliferative ($Ki67^+$) cells in the organoids developed from freshly biopsied and cryopreserved endometrial tissue treated without hormones. The median is shown and error bars display interquartile range. Proliferation did not differ significantly between fresh and frozen tissue-derived organoids. The following symbols correspond to respectively patient 1, 2 and 3: bullet, square, triangle.

between cryopreserved and freshly biopsied endometrial tissue. On day 7, organoid formation efficiency was 0.20% (0.11–0.19) for cryopreserved tissue and 0.62% (0.31–1.25) for freshly biopsied tissue ($P = 0.50$), whereas, on day 10, it was respectively 0.44% (0.29–0.46) and 1.24% (0.63–1.96) for cryopreserved and freshly biopsied tissue ($P = 0.75$) (FIGURE 1A). Organoid cultures from both fresh and frozen tissue could be similarly expanded and passaged for at least 13 passages (5 months) (FIGURE 1B; data not shown). Proliferative activity (as determined by the percentage of $Ki67^+$ cells) was not significantly different between fresh and cryopreserved tissue organoids (15.30% [14.21–16.74] versus 8.79% [7.57–10.41], respectively; $P = 0.25$) (FIGURE 1C).

Histological analysis confirmed organoid characteristics as previously described for fresh tissue-derived organoids (*Boretto et al., 2017*; *Turco et al., 2017*). Organoids from fresh and cryopreserved tissue showed a cystic structure with a central lumen lined by a single cell layer (FIGURE 2 and FIGURE 3). Organoids contained mucus in their lumen (as assessed by PAS staining, and expressed ERα, PR, PAEP (FIGURE 2 and FIGURE 3) and E-cadherin (FIGURE 4A and FIGURE 4B), thereby indicating the presence of secretory cells and pointing to polarization, together supporting a glandular phenotype.

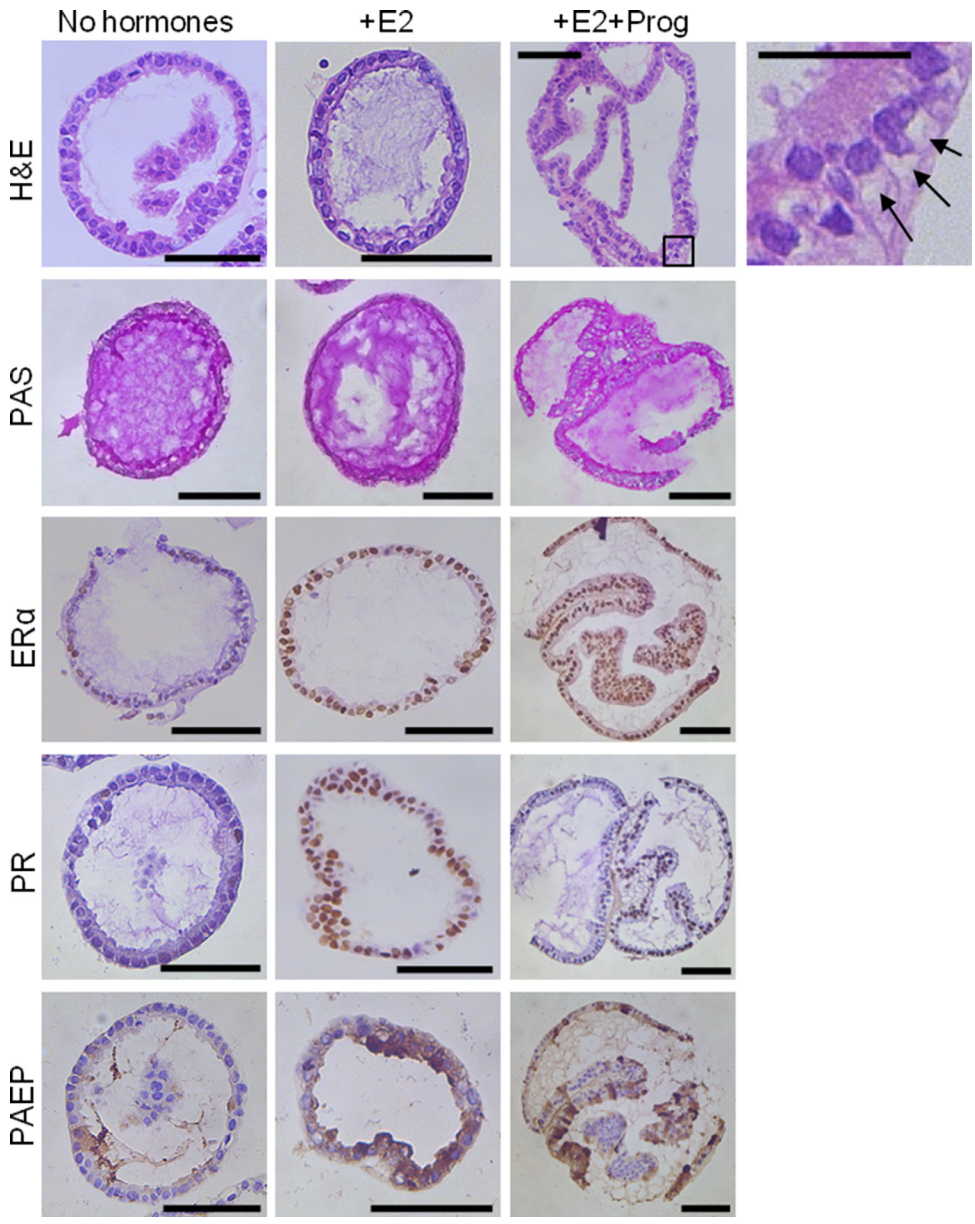


**FIGURE 2** Phenotypical characterization of freshly biopsied endometrial tissue-derived organoids and their hormonal responsiveness. All images were acquired using the 20x objective and cropped for better visualization of the organoid. Haematoxylin and eosin staining (H&E), showing cystic morphology, pseudostratified epithelium after oestradiol treatment and folded appearance with subnuclear glycogen vacuolation (inset [scale bar 20 μm] and arrows) in columnar epithelial cells after treatment with progesterone. Periodic acid-Schiff (PAS) staining displays the presence of mucus in the lumen of the organoids. Immunohistochemical staining of oestrogen receptor alpha (ERα) and progesterone receptor (PR), both upregulated after oestradiol treatment, and of progestagen-associated endometrial protein (PAEP), most upregulated after progesterone treatment. All scale bars except scale bar in inset: 50 μm.

Moreover, endometrial organoids derived from fresh and cryopreserved tissue showed responsiveness to oestradiol and progesterone (FIGURES 2–4). The morphological, immunophenotypical and ultrastructural hormone responses in these organoids were similar to those previously described for organoids derived from freshly biopsied endometrial tissue (*Boretto et al., 2017*). Transmission electron microscopy analysis ($n$ = 1) confirmed the presence of pseudostratified epithelium in oestradiol-treated endometrial organoids from cryopreserved tissue and demonstrated luminal microvilli (FIGURE 4C). After progesterone treatment, secretory vesicles, more pronounced microvilli and mucus were seen at the luminal surface, and cells contained glycogen deposits (FIGURE 4D). Moreover, apically located ciliated cells occurred (as demonstrated by acetylated α-tubulin immunoreactivity), further supporting the organoids' polarization (FIGURES 4C–4D).

Gene expression levels of endometrial markers in organoids, not treated with any hormones, derived from both fresh and cryopreserved tissue did not show any significant differences: *ESR1* ($P$ = 0.31), *FOXA2* ($P$ = 0.63), *LIF* ($P$ = 0.81), *MUC1* ($P$ = 0.06), *PAEP*

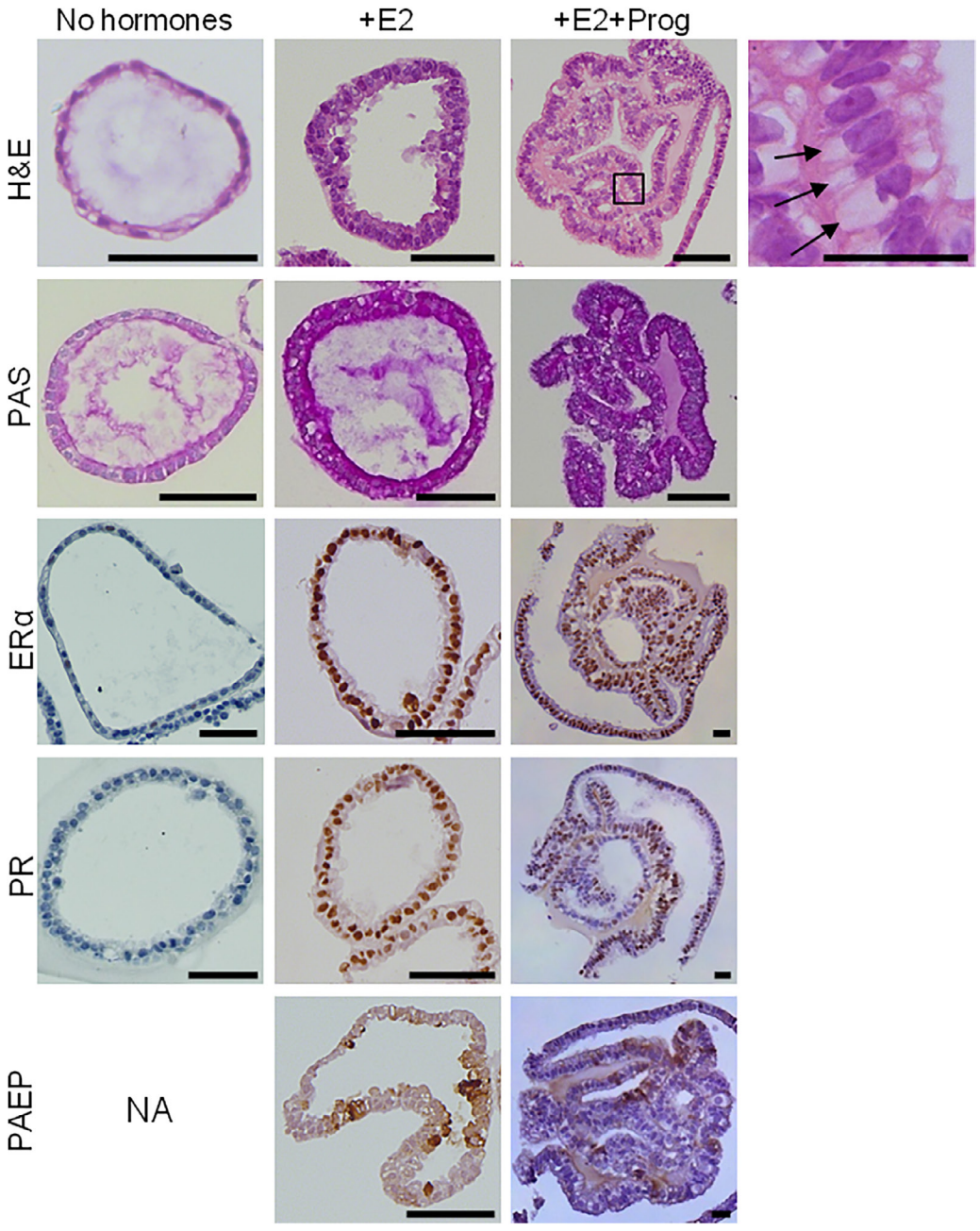


**FIGURE 3** Phenotypical characterization of cryopreserved endometrial tissue-derived organoids and their hormonal responsiveness. All images were acquired using the 20x objective and cropped for better visualization of the organoid. Haematoxylin and eosin staining (H&E), showing cystic morphology, pseudostratified epithelium after oestradiol treatment, and folded appearance with subnuclear glycogen vacuolation (inset [scale bar 20 μm] and arrows) in columnar epithelial cells after treatment with progesterone. Periodic acid-Schiff (PAS) staining, displaying the presence of mucus in the lumen of the organoids. Immunohistochemical staining of oestrogen receptor α (ERα) and progesterone receptor (PR), both upregulated after oestradiol treatment, and of progestagen-associated endometrial protein (PAEP), most upregulated after progesterone treatment. PAEP staining was not available (NA) for organoids treated without hormones. All scale bars except scale bar in inset: 50 μm.

(*P* = 0.63) and *PR* (*P* = 0.44) (FIGURE 5). Expression levels of endometrial markers after oestradiol treatment also did not differ significantly between organoids from both fresh and cryopreserved tissue: *ESR1* (*P* = 0.31), *FOXA2* (*P* = 0.81), *LIF* (*P* = 0.81), *MUC1* (*P* = 0.19), *PAEP* (*P* = 1.00), *PR* (*P* = 0.13) (FIGURE 5).

## DISCUSSION

The present study shows that organoids can be developed successfully from cryopreserved endometrial tissue. Moreover, they are comparable to organoids established from freshly biopsied tissue of the same patient. The morphology of the organoids and responses to hormonal treatment are in line with previous reports on organoids derived from freshly biopsied endometrial tissue of healthy women (*Boretto et al., 2017*; *Turco et al., 2017*) and confirm that organoids can also be reliably established from endometrial tissue obtained from infertile women.

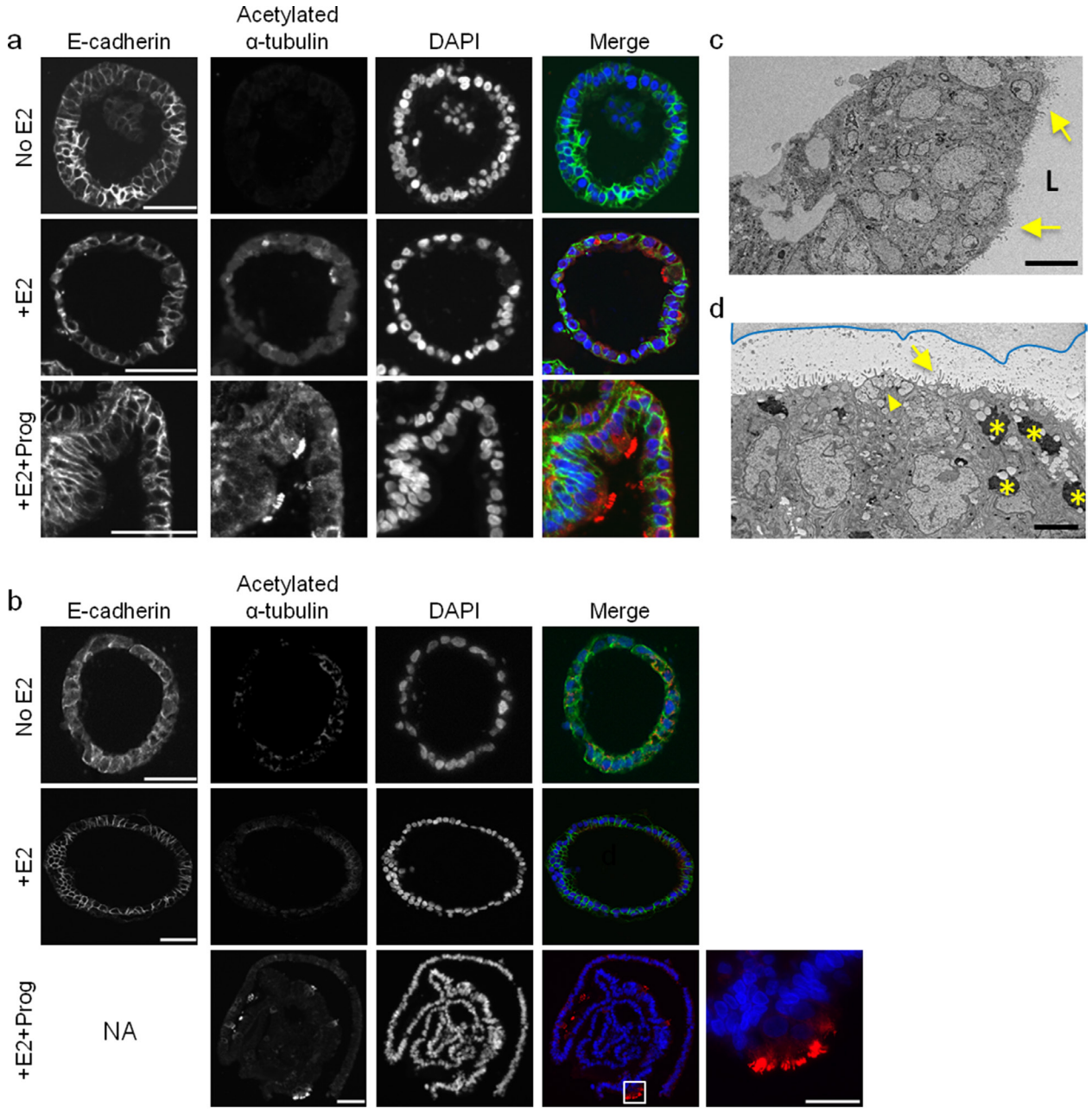


**FIGURE 4** Epithelial characterization and apicobasal polarity of freshly biopsied and cryopreserved endometrial tissue-derived organoids. (a–b) Immunofluorescence staining of the epithelial marker E-cadherin (green) and of acetylated α-tubulin (red) in endometrial organoids developed from (a) freshly biopsied tissue and (b) cryopreserved tissue, demonstrating the presence of ciliated cells in organoids treated with hormones. Nuclear staining was performed with DAPI (blue). E-cadherin staining was not available (NA) for organoids developed from cryopreserved tissue treated with progesterone. Scale bar in inset in (b) 20 µm, other scale bars in (a) and (b) 50 µm; (c) transmission electron microscopy (TEM) analysis of organoids treated with oestradiol developed from cryopreserved endometrial tissue, demonstrating apicobasal polarity with small microvilli (arrow) at the luminal surface (L), and pseudostratification of the columnar epithelium. Scale bar: 20 µm; (d) TEM analysis of organoids treated with progesterone developed from cryopreserved endometrial tissue, revealing the presence of secretory vesicles (yellow arrowhead) at the cell apex, glycogen deposits (yellow asterisks), more pronounced microvilli (yellow arrow) and mucus in the lumen (blue delineated area). Scale bar: 5 µm.

The processes of slow-freezing of tissue with standard cryoprotectant concentrations, i.e. 10% (DMSO) and subsequent thawing, are known to result in partial loss of cell viability (*Heng et al., 2006*). Along this line, apparently lower values for organoid formation efficiency of cryopreserved tissue compared with fresh tissue, were observed, although this difference was not significant and did not restrict long-term organoid expansion.

Prior cryopreservation of the endometrial tissue sample does not significantly influence organoid gene expression levels, as analysed for a number of endometrial markers. This observation is in line with a previous study exploring gastrointestinal organoids (*Tsai et al., 2018*). Organoid gene expression analysis also showed robust expression levels after treatment with oestradiol regardless of prior tissue cryopreservation.

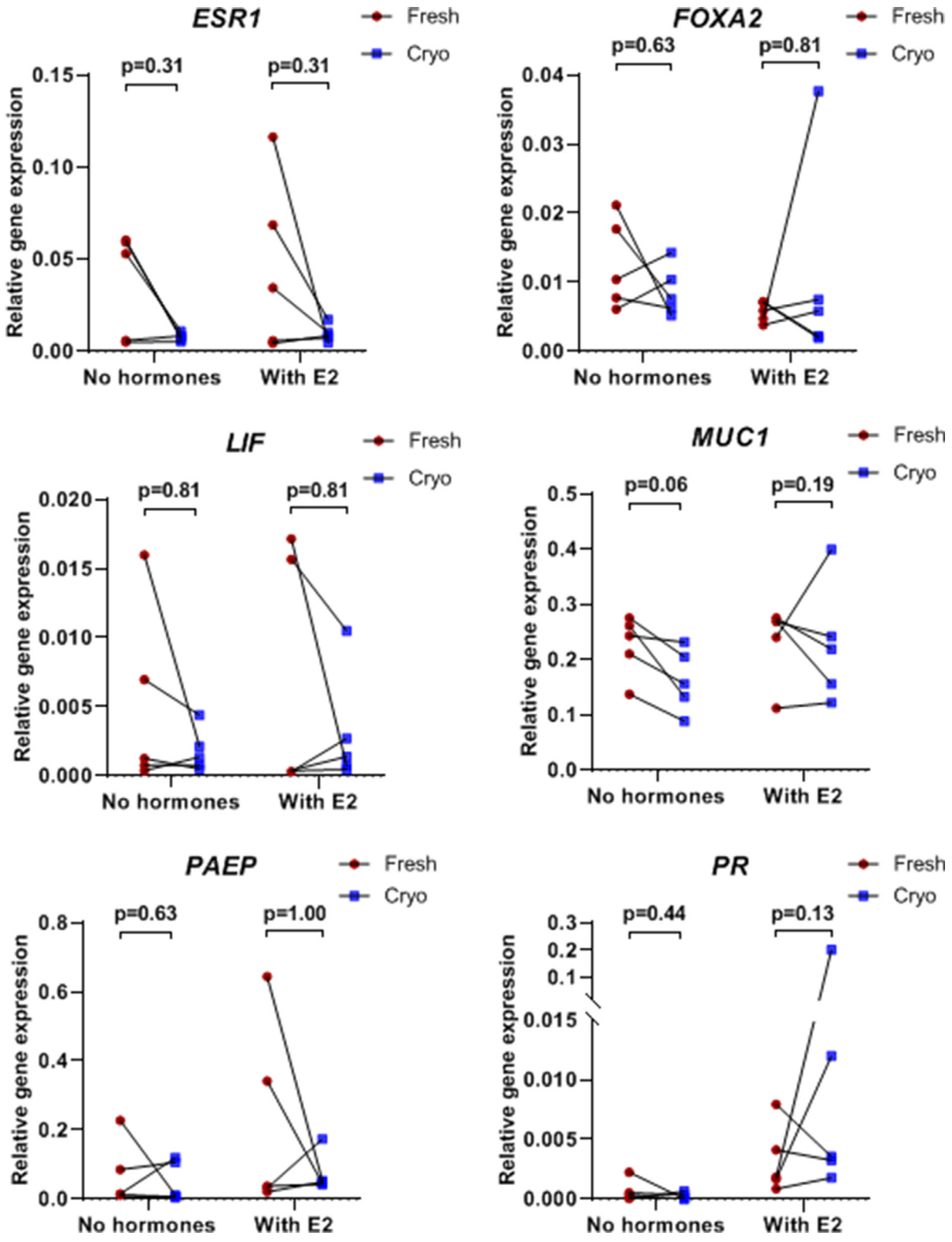


**FIGURE 5** Gene expression analysis of endometrial markers in endometrial organoids. Organoids are developed from freshly biopsied and cryopreserved endometrial tissue of five infertile women. Relative gene expression levels in organoids of each individual, normalized to reference gene *GAPDH*, are shown and analysed using a paired statistical test (Wilcoxon signed-rank test). The gene expression in freshly biopsied tissue-derived organoids is linked to the gene expression in the patient-matched cryopreserved tissue-derived organoids by a line.

An important strength of our study is that organoids from freshly biopsied and cryopreserved endometrial tissue, originating from the same patient, were compared, thereby excluding differences owing to interpatient variability. The primary aim of this study was to determine whether organoids could be developed from cryopreserved endometrial biopsies. As organoids were developed from infertile women, this will now allow endometrial aspects of infertility to be deciphered by comparing organoids in sufficiently large numbers (given certain interpatient variability) of infertile women to those developed from fertile women. Timed sampling of the endometrial tissue, i.e. in a specific cycle phase, may not be strictly required as previous studies (*Boretto et al., 2017*, *Turco et al., 2017*) have successfully developed organoids from (healthy) endometrium obtained during both the follicular and luteal phase, and since the organoids can reproduce the different cycle phases *in vitro* under defined hormonal treatment. Consequently, patient burden associated with performing urinary LH tests to time the biopsy can be reduced.

As the data were considered not normally distributed in a small sample size, statistical analysis was conducted using a non-parametric test, with inherently lower power compared with parametric statistical tests. The lack of statistical significance of the differences may, therefore, rather reflect the interpatient variability than differences owing to tissue cryopreservation. Interpatient variability was seen in organoid formation efficiency, although these differences did not limit long-term expansion of any organoid line. Variability was also seen in gene expression data, in which no significant differences were found. The variability can have various causes among a small sample size, which may mask true differences.

In conclusion, we have reliably established long-term expandable organoid lines from cryopreserved endometrial tissue obtained from infertile women. Our findings justify the use of cryopreserved tissue for development of endometrial organoids and are important for future organoid-based fertility research, which will no longer be limited to clinics situated near organoid laboratories. Furthermore, the robust expansion of endometrial organoids overcomes the previous tissue scarcity constraint and provides the opportunity to perform broad omics analysis. This may pave the way to unravelling the role of the endometrium in unexplained infertility and to new therapeutic strategies. In due course, the current empirical treatments that are regularly offered to infertile patients at high financial, emotional and physical costs may be replaced by targeted personalized medicine.

## ACKNOWLEDGEMENTS

The authors are grateful to the women who donated tissue for research. The randomized controlled trials are funded by The Netherlands Organisation for Health Research and Development, 'ZonMW' (ZonMW-project numbers 843002601 and 843001808). The project for development of organoids is funded by the De Snoo – van 't Hoogerhuijs Foundation. ZonMw and the De Snoo – van 't Hoogerhuijs Foundation are not involved in the study design, interpretation of data or writing of the manuscript.

## SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2020.03.019.

## REFERENCES

Boretto, M., Cox, B., Noben, M., Hendriks, N., Fassbender, A., Roose, H., Amant, F., Timmerman, D., Tomassetti, C., Vanhie, A., Meuleman, C., Ferrante, M., Vankelecom, H. **Development of organoids from mouse and human endometrium showing endometrial epithelium physiology and long-term expandability.** Development (Cambridge, England) 2017; 144: 1775–1786

Clevers, H. **Modeling Development and Disease with Organoids.** Cell 2016; 165: 1586–1597

Heng, B.C., Ye, C.P., Liu, H., Toh, W.S., Rufaihah, A.J., Yang, Z., Bay, B.H., Ge, Z., Ouyang, H.W., Lee, E.H., Cao, T. **Loss of viability during freeze-thaw of intact and adherent human embryonic stem cells with conventional slow-cooling protocols is predominantly due to apoptosis rather than cellular necrosis.** Journal of Biomedical Science 2006; 13: 433–445

Kodaman, P.H., Taylor, H.S. **Hormonal regulation of implantation.** Obstetrics and gynecology clinics of North America 2004; 31: 745–766

Kretzschmar, K., Clevers, H. **Organoids: Modeling Development and the Stem Cell Niche in a Dish.** Developmental cell 2016; 38: 590–600

Noyes, R.W., Hertig, A.T., Rock, J. **Dating the endometrial biopsy.** American Journal of Obstetrics and Gynecology 1975; 122: 262–263

Tsai, Y.H., Czerwinski, M., Wu, A., Dame, M.K., Attili, D., Hill, E., Colacino, J.A., Nowacki, L.M., Shroyer, N.F., Higgins, P.D.R., Kao, J.Y., Spence, J.R. **A Method for Cryogenic Preservation of Human Biopsy Specimens and Subsequent Organoid Culture.** Cellular and molecular gastroenterology and hepatology 2018; 6: 218–222

Turco, M.Y., Gardner, L., Hughes, J., Cindrova-Davies, T., Gomez, M.J., Farrell, L., Hollinshead, M., Marsh, S.G.E., Brosens, J.J., Critchley, H.O., Simons, B.D., Hemberger, M., Koo, B.K., Moffett, A., Burton, G.J. **Long-term, hormone-responsive organoid cultures of human endometrium in a chemically defined medium.** Nature cell biology 2017; 19: 568–577

van der Steeg, J.W., Steures, P., Eijkemans, M.J., Habbema, J.D., Hompes, P.G., Broekmans, F.J., van Dessel, H.J., Bossuyt, P.M., van der Veen, F., Mol, B.W. **CECERM study group (Collaborative Effort for Clinical Evaluation in Reproductive Medicine). Pregnancy is predictable: a large-scale prospective external validation of the prediction of spontaneous pregnancy in subfertile couples.** Human reproduction (Oxford, England) 2007; 22: 536–542

Walsh, A.J., Cook, R.S., Sanders, M.E., Arteaga, C.L., Skala, M.C. **Drug response in organoids generated from frozen primary tumor tissues.** Scientific reports 2016; 6: 18889

Received 16 October 2019; received in revised form 19 March 2020; accepted 27 March 2020.