Human Reproduction Open, Vol.00, No.0, pp. 1–13, 2021
doi:10.1093/hropen/hoab034

human reproduction open

ORIGINAL ARTICLE

A 3D endometrial organotypic model simulating the acute inflammatory decidualisation initiation phase with epithelial induction of the key endometrial receptivity marker, integrin αVβ3

R. Fraser *, R. Smith, and C.-J. Lin *

The University of Edinburgh, MRC Centre for Reproductive Health, Queen's Medical Research Institute, Edinburgh, UK

*Correspondence address. The University of Edinburgh, MRC Centre for Reproductive Health, Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ, UK. E-mail: rupsha.fraser@ed.ac.uk (R.F.) https://orcid.org/0000-0002-3847-3554; chih-jen.lin@ed.ac.uk (C.-J.L.) https://orcid.org/0000-0002-1234-4236

Submitted on September 18, 2020; resubmitted on June 22, 2021; editorial decision on August 23, 2021

STUDY QUESTION: Is it possible to develop a simplified physiological *in vitro* system representing the key cell-types associated with a receptive endometrial phenotype?

SUMMARY ANSWER: We present a new concept to investigate endometrial receptivity, with a 3D organotypic co-culture model to simulate an early and transient acute autoinflammatory decidual status that resolves in the induction of a receptive endometrial phenotype.

WHAT IS KNOWN ALREADY: Embryo implantation is dependent on a receptive uterine environment. Ovarian steroids drive post-ovulation structural and functional changes in the endometrium, which becomes transiently receptive for an implanting conceptus, termed the 'window of implantation', and dysregulation of endometrial receptivity is implicated in a range of reproductive, obstetric, and gynaecological disorders and malignancies. The interactions that take place within the uterine microenvironment during this time are not fully understood, and human studies are constrained by a lack of access to uterine tissue from specific time-points during the menstrual cycle. Physiologically relevant *in vitro* model systems are therefore fundamental for conducting investigations to better understand the cellular and molecular mechanisms controlling endometrial receptivity.

STUDY DESIGN, SIZE, DURATION: We conducted an *in vitro* cell culture study using human cell lines and primary human cells isolated from endometrial biopsy tissue. The biopsy tissue samples were obtained from three women attending gynaecological outpatient departments in NHS Lothian. The work was carried out between December 2016 and April 2019, at the MRC Centre for Reproductive Health, Queen's Medical Research Institute, University of Edinburgh.

PARTICIPANTS/MATERIALS, SETTING, METHODS: An endometrial stromal cell (ESC) line, and endometrial epithelial cells (EECs) isolated from endometrial biopsy tissue and expanded *in vitro* by conditional reprogramming, were used throughout the study. Immunocytochemical and flow cytometric analyses were used to confirm epithelial phenotype following conditional reprogramming of EECs. To construct an endometrial organotypic co-culture model, ESCs were embedded within a 3D growth factor-reduced Matrigel structure, with a single layer of conditionally reprogrammed EECs seeded on top. Cells were stimulated with increasing doses of medroxyprogesterone acetate, cAMP and oestradiol, in order to induce ESC decidual transformation and endometrial receptivity. Decidual response and the induction of a receptive epithelial phenotype were assessed by immunocytochemical detection and quantitative in-cell western analyses, respectively.

MAIN RESULTS AND THE ROLE OF CHANCE: A transient up-regulation of the interleukin-33 receptor protein, ST2L, was observed in ESCs, indicating a transient autoinflammatory decidual response to the hormonal stimulation, known to induce receptivity gene expression in the overlying epithelium. Hormonal stimulation increased the EEC protein levels of the key marker of endometrial receptivity,

integrin αVβ3 (n = 8; *$P < 0.05$; ***$P < 0.0001$). To our knowledge, this is the first demonstration of a dedicated endometrial organotypic model, which has been developed to investigate endometrial receptivity, via the recapitulation of an early decidual transitory acute autoinflammatory phase and induction of an epithelial phenotypic change, to represent a receptive endometrial status.

LIMITATIONS, REASONS FOR CAUTION: This simplified *in vitro* ESC-EEC co-culture system may be only partly representative of more complex *in vivo* conditions.

WIDER IMPLICATIONS OF THE FINDINGS: The 3D endometrial organotypic model presented here may offer a valuable tool for investigating a range of reproductive, obstetric, and gynaecological disorders, to improve outcomes for assisted reproductive technologies, and for the development of advances in contraceptive methods.

STUDY FUNDING/COMPETING INTEREST(S): This work was supported in part by a Medical Research Council Centre Grant (project reference MR/N022556/1). R.F. was the recipient of a Moray Endowment award and a Barbour Watson Trust award. C.-J.L. is a Royal Society of Edinburgh Personal Research Fellow, funded by the Scottish Government. The authors have no conflicts of interest to declare.

WHAT DOES THIS MEAN FOR PATIENTS?

During the first stage of the menstrual cycle, oestrogen secretion from the developing ovarian follicle promotes growth of the endometrium (the lining of the womb). Following ovulation, rising progesterone and oestrogen levels, produced by the corpus luteum (the remaining structure of the ovarian follicle that contained the maturing egg before its release during ovulation), promote structural and functional changes in the endometrium, in preparation for the 'window of implantation'—a period of 2–5 days when the endometrium is optimally receptive to an implanting embryo. This period of optimal endometrial receptivity is not only crucial for successful embryo implantation, but abnormal molecular and cellular events in the endometrium during this transient period have also been implicated in fertility problems, obstetric complications, gynaecological disorders, and endometrial cancer. In this study, we have developed a simplified cellular model, with physiologically appropriate hormonal stimulation, to investigate endometrial receptivity. A more comprehensive understanding of these events can lead to the development of new interventions to promote pregnancy success, long-term maternal and foetal health, women's health, as well as for improving contraceptive methods, and this new concept may be able to aid investigations to better understand the complex mechanisms involved in the generation of endometrial receptivity.

Key words: endometrial receptivity / window of implantation / decidualisation / endometrial organotypic model / conditional reprogramming / endometrial stromal cells / endometrial epithelial cells / progesterone / oestrogen

Introduction

Embryo implantation is a critical event in human pregnancy that is reliant on a receptive uterine environment. The cycling endometrium undergoes profound changes in women, leading to a carefully timed and defined period during which an embryo is able to attach and invade into a receptive uterus, resulting in the establishment of a successful pregnancy (Norwitz *et al.*, 2001). Ovarian steroids, oestrogen and progesterone, drive structural and functional changes in the uterine lining, preparing it for the implantation of a conceptus. The uterine lining, known as the endometrium, consists of a fibroblast-like stromal matrix lined by a single layer of columnar epithelium. Following ovulation, dynamic changes take place in the endometrial stromal cell (ESC) morphology, whereby ESCs undergo mesenchymal-to-epithelial transformation, and begin to differentiate into large, secretory, 'decidualised' stromal cells in response to rising progesterone levels produced by the corpus luteum (Gellersen *et al.*, 2007; Salamonsen *et al.*, 2009). Decidual transformation of ESCs is associated with enlargement and rounding of the nucleus, increased number of nucleoli, rough endoplasmic reticulum and Golgi complex expansion, and accumulation of glycogen and lipid droplets in the expanding cytoplasm (Gellersen and Brosens, 2014; Kajihara *et al.*, 2014; Okada *et al.*, 2018).

Decidualisation is a dynamic, multistep progression of events, comprising three critical transitory phases: an acute inflammatory initiation phase that subsequently transitions to an anti-inflammatory secretory phase during which time embryo implantation takes place, followed by a final resolution phase (Gellersen and Brosens, 2014). First, ESCs undergo cell cycle exit at G_0/G_1 and mount a transient pro-inflammatory response generated by a self-limiting autoinflammatory response, which, in turn, results in the expression of key receptivity genes in the overlying endometrial surface luminal epithelium (Salker *et al.*, 2012). This renders the endometrium receptive for embryo implantation for a limited period of time: the 'window of implantation'. The period of optimal endometrial receptivity begins ~6 days post-ovulation and lasts 2–5 days (i.e. approximately between days 20 and 25 of an idealised 28-day cycle) (Denker, 1993). A receptive endometrial phenotype is not only imperative for embryo implantation and pregnancy success, but aberrant decidual transformation and dysregulation of uterine receptivity have also been implicated in several obstetric complications, gynaecological disorders, and cancer (Norwitz, 2006; Strowitzki *et al.*, 2006; Cartwright *et al.*, 2010; Lessey, 2011; Patel and Lessey, 2011; Gellersen and Brosens, 2014; Timeva *et al.*, 2014; Rabaglino *et al.*, 2015; Tan *et al.*, 2015; Conrad *et al.*, 2017).

Interactions between different cell-types have reciprocal effects on cell phenotypes and ensuing functions (Freshney, 2005). The same is true for uterine compartments and the contributions of these interactions to endometrial receptivity, since decidual transformation of the stroma confers its ability to create paracrine gradients necessary for expression of evolutionarily conserved molecules by the luminal epithelium, which are fundamental for embryo implantation (Achache and Revel, 2006; Salker *et al.*, 2012). It has been demonstrated that endometrial receptivity is mediated by the activation of autoregulatory feedback loops in decidualising ESCs underlying the luminal epithelium, which activate the sequential expression of pro- and anti-inflammatory gene networks, and that ESCs can exert this function independent of local immune cells (Salker *et al.*, 2012). As such, it is evident that there is a co-dependent relationship between the endometrial stroma and epithelium to prepare the uterus for pregnancy (Cakmak and Taylor, 2011), with ESC decidual transformation being a prerequisite for the generation of endometrial receptivity (Vinketova *et al.*, 2016; Yu *et al.*, 2016). The current study focuses on the uterine phenotype during the acute inflammatory initiation phase of decidual transformation implicated in the generation of endometrial receptivity.

The mechanisms that control decidualisation and endometrial receptivity are highly complex, and we do not yet fully understand all the interactions that take place within the uterine microenvironment during this time. Uterine competence for embryo implantation sets the foundation for a successful pregnancy pathway; it is only when a clearer picture of the relative contributions of the cellular and molecular mechanisms leading to a receptive endometrial status becomes apparent, that the pathophysiology of several reproductive, obstetric, and gynaecological disorders can be further defined, and appropriate interventions can be developed to promote pregnancy success as well as long-term maternal and foetal health. Likewise, a better understanding of these mechanisms will also be beneficial for innovations in contraceptive methods. We have developed a simplified 3D endometrial organotypic model to investigate endometrial receptivity, in which we simulate an early acute inflammatory endometrial status that resolves in the generation of a receptive luminal epithelial phenotype, known as the 'window of implantation'. Organotypic culture refers to *in vitro* cell culture models in which two or more previously disaggregated cell-types are recombined in experimentally determined ratios and spatial relationships to reconstruct a constituent of the corresponding *in vivo* organ, as opposed to histiotypic cultures (high-density culture of a single cell-type within a 3D matrix) or organoid cultures (simplified, self-organising stem cell-derived 3D multicellular aggregates with the ability to mimic its *in vivo* organ counterpart) (Freshney, 2005; Simian and Bissell, 2017). An endometrial organotypic culture model, albeit a simplified representation, is able to better recapitulate the morphological and functional features of the *in vivo* uterine microenvironment than is possible using conventional 2D or even histiotypic cell culture methods. Physiologically representative *in vitro* model systems are vital for investigating the mechanisms implicated in endometrial receptivity, owing to the ethical and logistical limitations of human studies. While there are reports of several *in vitro* endometrial co-culture models in the literature (Bentin-Ley *et al.*, 2000; Arnold *et al.*, 2001; Bläuer *et al.*, 2005; Wang *et al.*, 2012; Chen *et al.*, 2013), these are not representative of the early acute inflammatory decidualisation phase that gives rise to a transiently receptive epithelial phenotype. The new functional co-culture system presented here may offer a convenient and accessible tool to improve our comprehension of interactions within the uterine microenvironment during this transitory phase.

Materials and methods

Culture and hormonal stimulation of the human ESC-derived cell line, St-T1b

The human ESC-derived telomerase-immortalised cell line, St-T1b (Samalecos *et al.*, 2009), kindly provided by Professor Jan Brosens (University of Warwick, UK), was maintained in phenol red-free Dulbecco's modified Eagle medium DMEM/Ham's F12 (DMEM/F12; Invitrogen, Renfrew, UK) with 10% steroid-depleted foetal calf serum (FCS) supplemented with 2 mM L-glutamine, 1 µg/ml insulin, 0.3 ng/ml 17β-oestradiol (E2), 50 µg/ml penicillin, 50 µg/ml streptomycin, and 0.2% Primocin (Invivogen, Toulouse, France) (ESC medium) at 37°C in an atmosphere of 5% CO_2. Phenol red-free medium was used in all experiments, due to phenol red's known oestrogenic activity (Berthois *et al.*, 1986). To induce decidualisation, cells were treated with minimal medium 1 (MM1; ESC medium without insulin and E2) containing increasing concentrations of the progestin, medroxyprogesterone acetate (MPA), 8-bromoadenosine 3',5'-cyclic adenosine monophosphate (8-Br-cAMP; Cambridge Bioscience, Cambridge, UK) and E2, or MM1 with 0.001% ethanol (Table I), every 48 h, and cultured over 8 days at 37°C in an atmosphere of 5% CO_2. All reagents for St-T1b cell culture were purchased from Sigma-Aldrich (Sigma-Aldrich, Dorset, UK) unless stated otherwise. Cultured cells were fixed in 4% paraformaldehyde (PFA) on days 4, 6, and 8 for immunocytochemical analysis.

Ethical approval and isolation of epithelial cells from endometrial biopsies

Primary human endometrial biopsy tissue was obtained from three women attending gynaecological outpatient departments in NHS

Table I Physiological stimulatory hormonal doses.

Timeline	Hormonal stimuli added with MM1	Control (unstimulated cells)
Day 0	0.25 µM MPA + 0.25 mM 8-Br-cAMP + 1 nM E2	MM1 + 0.001% EtOH
Days 2, 4, and 6	1 µM MPA + 0.5 mM 8-Br-cAMP + 10 nM E2	MM1 + 0.001% EtOH

St-T1b cells and human endometrial epithelial cells (in monoculture or within the 3D endometrial organotypic model) were stimulated with minimal medium 1 (MM1) containing medroxyprogesterone acetate (MPA), 8-bromoadenosine 3′,5′-cyclic adenosine monophosphate (8-Br-cAMP) and 17β-oestradiol (E2), or MM1 with 0.001% ethanol (EtOH), every 48 h, and monitored over 8 days.

Lothian. Written informed consent was obtained from participants and ethical approval granted from Lothian Research Ethics Committee (REC 16/ES/0007). The women reported regular menstrual cycles and did not have exogenous hormone exposure for 2 months prior to biopsy. Women receiving hormonal therapy, suffering from endometriosis or those with fibroids of >3 cm were excluded. Human endometrial epithelial cells (EECs) were isolated from endometrial biopsies by tissue digestion and separation from ESCs. Endometrial tissue was minced using scalpel blades, followed by digestion with 100 μg/ml collagenase II and 0.25 μg/ml DNAse I (Sigma-Aldrich, Dorset UK) for 1.5 h at 37°C. The tissue homogenate was then sequentially strained through 70 and 40-μm membrane filters to separate glandular epithelium from ESCs. The membrane filters were back-washed with PBS to retrieve endometrial gland clumps, further rinsed with PBS to flush out any digestion medium, and mixed well to disperse clumps. EECs were then suspended in PBS and centrifuged at 500*g* for 5 min at room temperature, supernatant subsequently discarded, followed by resuspension in PBS and centrifugation at 500*g* for 5 min at room temperature.

Expansion of EEC by conditional reprogramming

EECs were rapidly expanded *in vitro* by conditional reprogramming with the use of Y-27632 (a Rho kinase inhibitor) and fibroblast feeder cells. First, 3T3 Swiss Albino fibroblasts (cell line obtained from the European Collection of Authenticated Cell Culture, Public Health England, Salisbury, UK) were grown in MM1 to ~80% confluence in T175 flasks, trypsinated, washed, resuspended in MM1 and irradiated at 30 Gy. The irradiated cells were washed, cultured at 37°C in an atmosphere of 5% CO_2, and conditioned medium collected 72 h post-irradiation. EEC medium was prepared with phenol red-free DMEM/Ham's F12 containing 10% steroid-depleted FCS, and supplemented with 2 mM L-glutamine, 5 μg/ml insulin, 24 μg/ml adenine, 0.4 μg/ml hydrocortisone, 10 ng/ml epidermal growth, 8.4 ng/ml cholera toxin, 10 μmol/l Y-27632 (Cambridge Bioscience, Cambridge, UK), 10 μg/ml gentamycin and 0.25 μg/ml amphotericin. The irradiated 3T3 conditioned medium was added to EEC medium in a 1:3 ratio, one part of IR 3T3 conditioned media to three parts of EEC medium; CREEC medium (conditional reprogramming EEC medium), and the EECs maintained in CREEC medium at 37°C in an atmosphere of 5% CO_2. This method has previously been shown to directly alter cell growth without selecting for a small sub-population of stem-like cells, while retaining a normal non-tumourigenic karyotype, and conditionally inducing an indefinite proliferative state in primary mammalian epithelial cells (Liu *et al.*, 2012; Suprynowicz *et al.*, 2012; Palechor-Ceron *et al.*, 2013). All reagents for EEC culture and conditional reprogramming were purchased from Sigma-Aldrich (Sigma-Aldrich, Dorset UK) unless stated otherwise. EECs can be cryopreserved using mFreSR™ cryopreservation medium (Stemcell Technologies, Cambridge, UK). Cultured EECs were fixed in 4% PFA for immunocytochemical analyses.

Immunocytochemical confirmation of ESC decidualisation and EEC phenotype

Fixed cells (St-T1b cells and EECs) were permeabilised with 0.1% Triton X-100 in PBS for 10 min, and washed three times with 0.1% Tween-20 in PBS (PBST). Cells were then blocked with 5% bovine serum albumin in PBS for 1 h at room temperature, incubated with primary antibody overnight at 4°C, washed three times with 0.1% PBST, subsequently incubated with secondary antibody and 1:10 000 DAPI for 30 min at room temperature in the dark, followed by a final wash with PBS. Primary antibodies used were rabbit anti-human insulin-like growth factor-binding protein-1 (IGFBP-1; Abcam, Cambridge, UK; ab111203; 1:100), rat anti-mouse ST2L (IL-33R/ST2) (eBioscience, Cheshire, UK; 17-9335-82; 1:100), rabbit anti-human cytokeratin-18 conjugated to phycoerythrin (Abcam, Cambridge, UK; ab218288; 1:1000), rabbit anti-human vimentin (New England Biolabs, Hitchin, UK; 5741; 1:100), and mouse anti-human integrin αVβ3 (Abcam, Cambridge, UK; ab190147; 1:100). A goat anti-rabbit antibody conjugated to Alexa Fluor 546 (Invitrogen, Renfrew, UK; A-11071; 1:300), a donkey anti-rabbit antibody conjugated to Alexa Fluor 568 (Invitrogen, Renfrew, UK; A10042; 1:250) and a donkey anti-mouse antibody conjugated to Alexa Fluor 488 (Invitrogen, Renfrew, UK; A21202; 1:500) were used as secondary antibodies. The cytokeratin-18 staining did not require incubation with a secondary antibody. The ST2L staining did not require cell permeabalisation for detection, but did require a signal amplification step after primary antibody incubation, with a biotinylated goat anti-rat antibody (Vector Laboratories, Peterborough, UK; BA-4000; 1:100) for 30 min at room temperature in the dark, followed by three washes with 0.1% PBST. Cells were then incubated with streptavidin conjugated to Alexa Fluor 488 (Invitrogen, Renfrew, UK; s11223; 1:200) and 1 mg/ml Hoechst 33342 (Invitrogen, Renfrew, UK; H3570) for 30 min at room temperature in the dark, and then washed with PBS. Imaging was conducted on an Olympus IX71 microscope with a QImaging optiMOS camera and CoolLED PE4000 light source (Olympus, Tokyo, Japan) or a Zeiss Axio Observer 7 microscope (Carl Zeiss Ltd, Cambridge, UK) with a Hamamatsu ORCA-Flash LT camera (Hamamatsu Protonics, Hertfordshire, UK) and Zeiss Colibri 7 LED light source (Carl Zeiss Ltd, Cambridge, UK). Images were analysed using ImageJ software (ImageJ, US National Institutes of Health, Bethesda, MD, USA).

Flow cytometric confirmation of EEC phenotype

EECs were blocked with 10% normal goat serum for 10 min on ice. Cells were then either left unstained (negative control) or incubated with mouse anti-human E-cadherin-BV421 (BD Biosciences, Oxford, UK), mouse anti-human epithelial cell adhesion molecule (EpCAM)-PE (Abcam, Cambridge, UK), mouse anti-human CD31-PerCP-Cy5.5, and rat anti-human CD45-APC-Cy7 (BioLegend, London, UK). Flow cytometry was carried out on a BD LSR Fortessa 5 L flow cytometer (BD Biosciences, Oxford, UK). Analysis was carried using FlowJo software (BD Biosciences, Oxford, UK).

Generation of a 3D *in vitro* organotypic model of a receptive endometrium

EECs were primed in ESC medium (containing 1 μg/ml insulin and 0.3 ng/ml E2) for 48 h at 37°C in an atmosphere of 5% CO_2. When the EECs had already been incubating with ESC medium for 24 h, St-T1b cells were seeded at a ratio of 1:3 growth factor-reduced (GFR) Matrigel (BD Biosciences, Oxford, UK) in ESC medium, at a density of

6×10^6 cells/ml, 60 µl/well (3.6×10^5 cells/well) in wells of a 96-well plate, and allowed to set into a 3D structure at 37°C in an atmosphere of 5% CO_2 over 45 min. A further 200 µl ESC medium was subsequently added to wells and maintained overnight at 37°C in an atmosphere of 5% CO_2. Once the EECs had been primed in ESC medium for 48 h, and the St-T1b cells grown within GFR Matrigel overnight, the medium was removed from wells containing the St-T1b 3D structures, and EECs were trypsinised, washed, resuspended in ESC medium and seeded on top of the 3D St-T1b cells at a density of 1×10^7 cells/ml, 100 µl/well (1×10^6 cells/well). To confirm that the phenotypic changes resulting from the hormonal stimulation were dependent on cell-to-cell communication between the stromal and epithelial compartments in our model, a parallel group was included in which EECs were cultured alone, without ESCs, on GFR Matrigel-coated plates. Cells were further incubated overnight at 37°C in an atmosphere of 5% CO_2. Following overnight incubation, hormonal stimuli were added to cells every 48 h as described in Table I, with the first addition of stimuli considered as day 0. Cells were fixed with 4% PFA on days 4, 6, and 8 for quantitative in-cell western analyses.

Quantification of integrin αVβ3 expression by in-cell western assay

Fixed cells were blocked overnight with Odyssey® buffer (LI-COR Biosciences, Cambridge, UK), followed by incubation with mouse anti-human integrin αVβ3 (Abcam, Cambridge, UK; ab190147; 1:100), overnight at 4°C. Cells were then washed with PBS and the subsequent protocol, using a goat anti-mouse IRDye® 800CW antibody and the CellTag™ 700 normalisation stain (LI-COR Biosciences, Cambridge, UK), was carried out according to the manufacturer's instructions. Cells were imaged and analysed using the Odyssey CLx Near-Infrared Fluorescence Imaging System (LI-COR Biosciences, Cambridge, UK). In each experiment, data were normalised such that the integrin αVβ3 expression in the control (unstimulated) wells were given a value of 100.

Statistical analysis

One-way ANOVA with Tukey's multiple comparison post-test was used to determine *P* values using GraphPad Prism (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

Results

Decidualisation induces the transient up-regulation of ST2L in St-T1b cells

Decidualisation was induced in St-T1b cells with increasing levels of MPA, E2, and cAMP over 8 days (Table I), to model the rising progesterone and oestrogen levels that drive the structural and functional changes in the secretory stage endometrium. Transformation of the St-T1b cells into characteristically larger, rounded 'decidualised' stromal cells, with increased cytoplasmic and nuclear size, was observed. Decidualisation was confirmed by immunocytochemical analyses of IGFBP-1 expression (Fig. 1A). IGFBP-1 is a widely used marker to assess the differentiation status and quality of the decidual response of ESCs in culture (Gao *et al.*, 1994; Giudice *et al.*, 1998; Fazleabas *et al.*, 2004; Kim *et al.*, 2007; Samalecos *et al.*, 2009; Gellersen and Brosens, 2014; Tamura *et al.*, 2018). Furthermore, decidual transformation of ESCs was additionally corroborated by visible enlargement and rounding of the nucleus and an expanding cytoplasm upon hormonal stimulation, which also induced a transient up-regulation of the interleukin-33 (IL-33) transmembrane receptor, ST2L, on day 6 (Fig. 1B), indicating a transient autoinflammatory decidual response.

Primary human EECs were rapidly expanded *in vitro* by conditional reprogramming, with retention of epithelial markers

Epithelial cells can be rapidly expanded *in vitro* by conditional reprogramming, with the use of a Rho kinase inhibitor (Y-27632) and irradiated fibroblast feeder cells (Liu *et al.*, 2012; Suprynowicz *et al.*, 2012; Palechor-Ceron *et al.*, 2013). This technique was adapted for human EECs in the current study, following isolation from clinical endometrial biopsies via tissue digestion with collagenase and DNase, and separation from ESCs (Fig. 2A). Conditionally reprogrammed EECs were generated, and these cells could be passaged several times with retention of epithelial markers. The conditionally reprogrammed EECs could be cryopreserved in single-cell suspension in mFreSR™ freezing medium and successfully revived following cryopreservation. EEC phenotype was confirmed by immunocytochemical expression of the epithelial marker, cytokeratin-18, and absence of the stromal cell marker, vimentin (Fig. 2B). Further validation of an epithelial phenotype was conducted by flow cytometric analyses, which demonstrated that the cells expressed two additional epithelial markers, EpCAM and E-cadherin, but did not express the leukocyte and endothelial cell markers, CD45 and CD31, respectively (Fig. 2C).

Generation of a novel endometrial organotypic *in vitro* co-culture model of the 'window of implantation'

First, EECs and ESCs were primed in medium containing E2 and insulin to model the proliferative stage of the uterine cycle. EECs were then co-cultured with St-T1b cells in a 3D structure (Fig. 3A), to produce an endometrial organotypic co-culture model. The organotypic 3D co-cultures were subjected to decidualisation hormonal stimuli over 8 days (Table I), to model the secretory stage of the uterine cycle and ultimately a receptive endometrial phenotype. EECs were monitored for expression of integrin αVβ3 (a key marker of uterine receptivity) by quantitative immunocytochemical detection, and in-cell western analyses demonstrated that integrin αVβ3 expression by EECs was significantly higher on day 8 after hormonal stimulation compared to basal expression where the cells did not receive any hormonal stimuli ($P < 0.0005$), as well as in comparison to integrin αVβ3 expression on day 4 of treatment ($P < 0.05$) (Fig. 3B; Supplementary Tables SI and SII). There was no significant change over time in basal integrin αVβ3 expression in the control unstimulated group, and the data from the stimulated cells were therefore normalised to the control unstimulated group. Furthermore, there was no induction of epithelial αVβ3 expression in a parallel group in which EECs were cultured alone and subjected to hormonal stimuli over 8 days (Supplementary Fig. S1B), likely

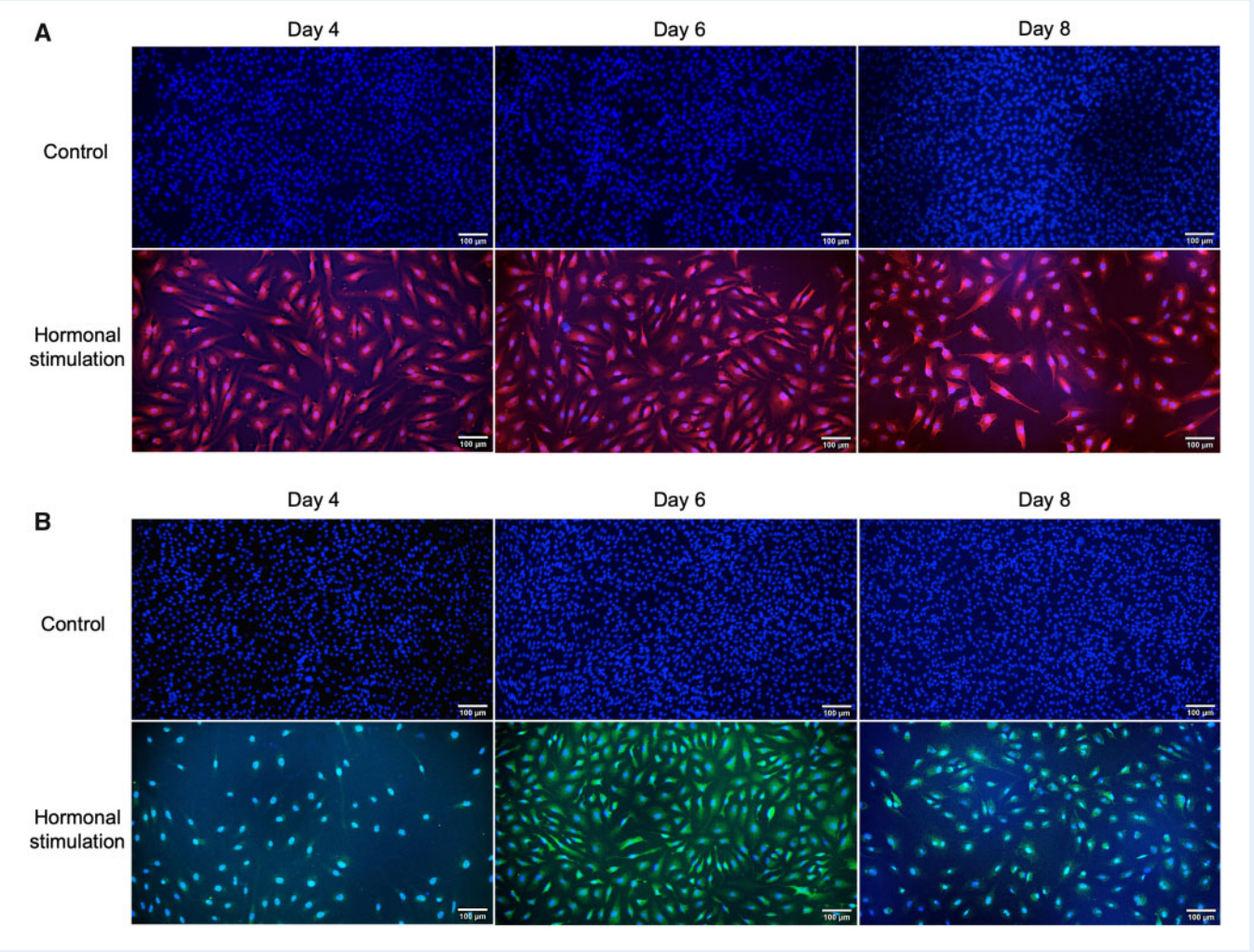

Figure 1. Assessment of decidualisation following stimulation with physiological stimulatory hormonal doses. Hormonal stimulation of the human endometrial stromal cell-derived, telomerase-immortalised St-T1b cell line with medroxyprogesterone acetate, 8-bromoadenosine 3′,5′-cAMP and 17β-oestradiol. Decidualisation was confirmed by immunohistochemical detection of (**A**) insulin-like growth factor-binding protein-1 (IGFBP-1) expression, and (**B**) the transient up-regulation of ST2L (the interleukin-33 receptor) levels. Scale bars = 100 μm.

indicating combined effects of hormone treatment and cell-to-cell communication between the stromal and epithelial compartments in our model.

Discussion

Decidualisation begins during the secretory phase of the menstrual cycle in response to rising steroid hormone levels, and is marked by the differentiation of fibroblast-like ESCs into specialised decidual cells, secretory changes in the uterine epithelial glands, the accumulation of uterine natural killer cells, and vascular changes in the uterine spiral arteries (Maruyama and Yoshimura, 2008; Cartwright *et al.*, 2010; Fraser *et al.*, 2015). These changes are not only important for implantation success, but defective endometrial receptivity is also associated with a wide range of gynaecological, reproductive, and obstetric disorders, as well as the pathophysiology of reproductive malignancies (Makieva *et al.*, 2018).

The key molecular players of ESC decidual transformation are progesterone and cAMP, which act synergistically to stimulate successful differentiation of ESCs into their decidualised state (Brar *et al.*, 1997; Gellersen and Brosens, 2003). Progesterone acts on ESCs by binding to the progesterone receptor (PR), a member of the steroid/thyroid hormone receptor superfamily of ligand-activated transcription factors (Gellersen and Brosens, 2003). Oestrogen is responsible for inducing PR expression in ESCs, which determines progesterone responsiveness during the secretory stage of the uterine cycle (Patel *et al.*, 2015). Rising progesterone levels drive the structural and biochemical changes from proliferative to secretory ESC status, with a simultaneous generation of endometrial receptivity and opening of the 'window of implantation' (Paulson, 2011), and activation of the cAMP second messenger pathway can direct cellular specificity to progesterone action through the induction of diverse transcription factors that affect PR function

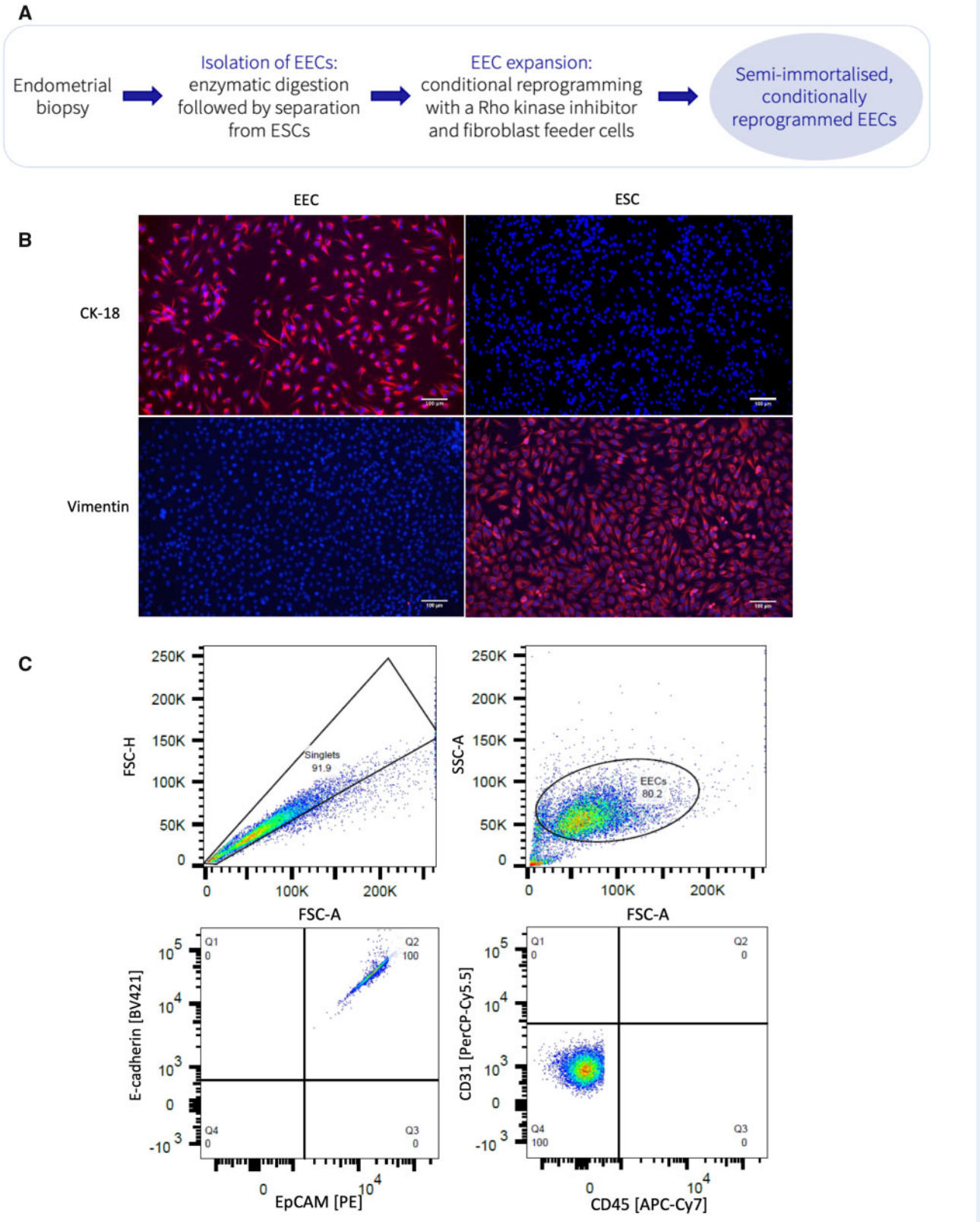

Figure 2. Conditional reprogramming of endometrial epithelial cells and confirmation of epithelial phenotype. (**A**) Endometrial epithelial cells (EECs) were isolated from endometrial biopsies and expanded *in vitro* by conditional reprogramming. Confirmation of epithelial phenotype by (**B**) immunocytochemical analyses demonstrated cytokeratin-18 (CK-18) expression and negative vimentin staining in EECs, with endometrial stromal cells (ESCs) serving as negative and positive controls, respectively. Scale bars = 100 μm. (**C**) Flow cytometric analyses demonstrated epithelial cell adhesion molecule (EpCAM) and E-cadherin expression by EECs, but no CD31 and CD45 expression.

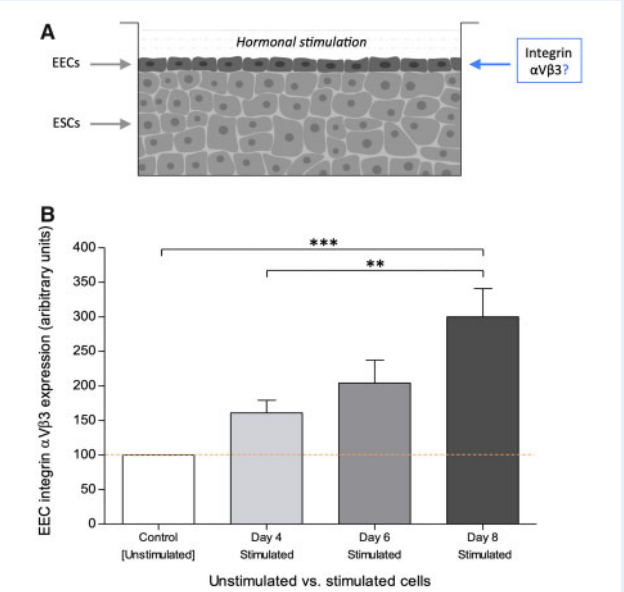

Figure 3. Assessment of epithelial integrin αVβ3 expression following stimulation with physiological stimulatory hormonal doses. (**A**) To construct a 3D endometrial organotypic co-culture model, St-T1b cells were embedded in growth factor-reduced Matrigel, with a single layer of EECs seeded on top. (**B**) In-cell western analysis was conducted to quantify epithelial integrin αVβ3 expression with or without treatment with medroxyprogesterone acetate, 8-bromoadenosine 3′,5′-cyclic adenosine monophosphate, and 17β-oestradiol, on days 4, 6, and 8. Results are mean ± SEM of eight separate experiments. $^{**}P < 0.05$; $^{***}P < 0.0001$; one-way ANOVA with Tukey's multiple comparison post-test analysis.

(Gellersen and Brosens, 2003). The initiation of endometrial receptivity is dependent on the local removal of steroid action in the endometrial epithelium, facilitated via selective down-regulation of epithelial PRs and oestrogen receptors (ERs), combined with the steroid-mediated paracrine effects from the stromal compartment. Through the selective epithelial cell PR and ER down-regulation, it is believed that progesterone and oestrogen act on stromal cells, which then influence epithelial cells through specific paracrine factors (Lessey *et al.*, 1996; Lessey, 1998). However, while adequate progesterone signalling is necessary to establish a receptive endometrial status, some studies suggest that untimely and/or excessive progesterone levels can compromise decidualisation and endometrial receptivity (Labarta *et al.*, 2011, Liang *et al.*, 2018). Furthermore, while progesterone is responsible for the structural ESC changes during decidualisation, animal studies have demonstrated that uterine oestrogen biosynthesis is also crucial for the progression of decidualisation, possibly by promoting stromal cell gap junction communication that is known to be involved in endometrial preparation for implantation (Ma *et al.*, 2003; Das *et al.*, 2009). Moreover, studies in mice have shown that oestrogen is critical in regulating the receptive endometrial state; low oestrogen levels can extend the 'window of implantation', whereas disproportionately high oestrogen levels can promptly initiate a refractory state, indicating that a narrow range of oestrogen levels can determine the duration of endometrial receptivity (Ma *et al.*, 2003), which could have implications in the human setting. Ovarian hormonal signalling must therefore be stringently regulated to establish an adequately programmed, appropriately timed receptive uterine environment to ensure pregnancy success, and to maintain gynaecological and reproductive health.

In the present study, decidualisation was induced with increasing doses of MPA, 8-Br-cAMP, and E2 over 8 days, in order to recapitulate the time it takes for these functional changes to occur *in vivo*, since the 'window of implantation' becomes apparent (through detection of epithelial integrin αVβ3 expression) 6–8 days after ovulation (Lessey, 1998). Frequently used *in vitro* decidualisation protocols include treatment of ESCs with constant doses of various combinations of progesterone or a progestin, a cAMP-inducing analogue and E2, with high variability in duration of treatment (Logan *et al.*, 2013; Gellersen and Brosens, 2014; Michalski *et al.*, 2018). Early *in vitro* decidualisation studies established that progestins (such as MPA) induce enhanced decidual effects in cultured ESCs compared to progesterone, that a combination of progesterone with E2 can amplify decidual effects in ESCs compared to treatment with progesterone alone, and that the cAMP signal transduction cascade is a key stimulant in progesterone-dependent decidualisation (Eckert and Katzenellenbogen, 1981; Irwin *et al.*, 1989; Levin *et al.*, 1990; Gellersen *et al.*, 1994; Brar *et al.*, 1997). Observations were based on physiological doses of ovarian hormones and cAMP stimulation that induced ESC ultrastructural and molecular changes characteristic of *in vivo* decidualisation (Eckert and Katzenellenbogen, 1981; Irwin *et al.*, 1989; Gellersen *et al.*, 1994). While the majority of *in vitro* decidualisation protocols make use of continuous hormonal stimulatory doses (Logan *et al.*, 2013; Gellersen and Brosens, 2014; Michalski *et al.*, 2018), we used increasing doses of hormonal and cAMP stimulation, to better represent the *in vivo* post-ovulatory rise in ovarian hormones and local cAMP production that controls decidualisation and endometrial receptivity. Our study demonstrates that these rising levels of ovarian hormones and cAMP can induce the transient up-regulation of the IL-33 receptor, ST2L, which was not observed when ESCs were subjected to the standard continuous doses of hormonal and cAMP stimulation reported in the literature (Logan *et al.*, 2013; Gellersen and Brosens, 2014; Michalski *et al.*, 2018) (Supplementary Fig. S1). Decidualising ESCs induce a transitory, acute autoinflammatory response, through secretion of IL-33, a key regulator of the innate immune response, while up-regulating the expression of its receptor, ST2L. This IL-33–ST2L signalling induces receptivity gene expression in the overlying epithelium, rendering the endometrium transiently receptive for the implantation of a conceptus (Salker *et al.*, 2012). In the absence of an implanting conceptus, the ESCs subsequently mount an anti-inflammatory response that involves the down-regulation of ST2L (Salker *et al.*, 2012).

Human studies are restricted by a lack of access to tissue throughout the different stages of the menstrual cycle. The current study provides a simple *in vitro* organotypic co-culture model of a 3D uterine structure, using Matrigel, an ESC cell line, and conditionally reprogrammed EECs. Matrigel is rich in laminin and collagen, bearing similarities to the uterine extracellular matrix composition (Tarrade *et al.*, 2002). Furthermore, it has been suggested that in addition to inducing a stromal regulatory phenotype, Matrigel is able to act as a mediator for ESCs to signal to EECs in a similar paracrine manner to what

occurs in living uterine tissue, upon ESC-EEC co-culture with Matrigel serving as the basement membrane (Arnold *et al.*, 2001). The St-T1b cell line used in our study expresses phenotypic ESC markers and can mimic primary decidual stromal cell responses *in vitro* (Samalecos *et al.*, 2009): its use eliminates patient variability, as well as the possibility of 'contaminating' EECs being present within the stromal ESC component of the model. However, an EEC cell line was not utilised, as all commonly used EEC cell lines are derived from malignant endometrial adenocarcinoma tissues. Since cancer cells have undergone numerous genetic and epigenetic alterations, adenocarcinoma-derived cell lines are not representative of non-cancerous biological processes such as decidualisation and the induction of endometrial receptivity. Conditional reprogramming transcends the difficulty of growing primary EECs in long-term culture, but allows propagation of primary epithelial cells into a highly proliferative state while cells maintain their original karyotype and remain in a non-neoplastic state (Liu *et al.*, 2012; Suprynowicz *et al.*, 2012; Palechor-Ceron *et al.*, 2013). Cells are denoted as 'conditionally reprogrammed' because of the conditional induction of cell proliferation with increased telomerase expression, by a combination of Y-27632 (which suppresses differentiation and extends life span in calcium- and serum-containing medium) and diffusible factor(s) released by the irradiation-induced apoptotic 3T3 feeder cells (Suprynowicz *et al.*, 2012; Palechor-Ceron *et al.*, 2013). It has been suggested that the unrestricted cell proliferation induced by conditional reprogramming is mediated through the induction of telomerase and cytoskeletal remodelling and/or interference with the p16/Rb pathway (Liu *et al.*, 2012; Palechor-Ceron *et al.*, 2013). The capacity for rapid establishment of karyotype-stable cell cultures from normal human epithelium facilitates *in vitro* cellular studies without the drawbacks of cell cultures generated, for example, from induced pluripotent stem cells (such as genetic instability, tumourigenicity, and altered antigenicity) (Suprynowicz *et al.*, 2012). Large numbers of EECs were generated through conditional reprogramming in the present study, which could be passaged multiple times with the retention of epithelial markers, thus providing the advantages of a conventional cell line.

The luminal epithelium is perceived as the fundamental site for endometrial receptivity (Idelevich and Vilella, 2020), and integrin αVβ3 is a cell-surface adhesion receptor that appears on the apex of endometrial luminal epithelial cell surfaces, coincident with the 'window of implantation', and has putative roles in embryo attachment during implantation (Rai *et al.*, 1996; Apparao *et al.*, 2001; Lessey, 2002; Lessey and Castelbaum, 2002). Integrin αVβ3 is maximally expressed during the 'window of implantation' (Apparao *et al.*, 2001), and its endometrial expression is significantly lower in cases of unexplained infertility, indicating that aberrant epithelial integrin αVβ3 expression may be associated with defective endometrial receptivity (Elnaggar *et al.*, 2017). Here, we capitalise on the acute inflammatory initiation phase of decidual transformation that promotes the generation of endometrial receptivity. This temporal endometrial phenotypic change is not only important for implantation success, but its dysregulation is also associated with a wide range of gynaecological, reproductive, and obstetric disorders, as well as in the pathophysiology of reproductive malignancies (Makieva *et al.*, 2018). Endometrial receptivity is mediated through both direct and indirect progesterone action (Lessey, 2003). Epithelial steroid receptor expression varies during the menstrual cycle, with high PR levels in the proliferative phase and selective loss of epithelial PR (and reduced ER) expression in the secretory phase (Lessey *et al.*, 1996), demonstrating a direct action of progesterone on epithelial cells. Endometrial receptivity is tightly associated with the shifts in PR and ER expression, which occur at the time of its onset around 5–6 days post-ovulation, concomitant with the appearance of epithelial integrin αVβ3 (Lessey, 1998). Stromal cells, on the other hand, maintain their PR expression throughout the menstrual cycle, and progesterone action on stromal cells generates paracrine mediators (such as the secretion of specific growth factors, cytokines, and inflammatory mediators) (Al-Sabbagh *et al.*, 2011; Salker *et al.*, 2012; Chen *et al.*, 2013) that promote epithelial gene expression, indicating the indirect action of progesterone, via stromal cells, in the induction of an epithelial receptive phenotype (Lessey, 1998; Lessey, 2003; Salker *et al.*, 2012). The addition of hormonal stimuli to our ESC-EEC co-culture system induced an autoinflammatory decidual stromal response and the up-regulation of epithelial integrin αVβ3, representing phenotypic endometrial changes concurrent with the 'window of implantation'.

Epithelial integrin αVβ3 expression within our 3D co-culture model coincided with the timing of the transient ST2L up-regulation that was observed in ESCs and was subsequently further amplified. Furthermore, there was no induction of epithelial integrin αVβ3 expression when EECs were cultured alone, without ESCs, and subjected to hormonal stimulation (Supplementary Fig. S1B). This suggests that the induction of epithelial integrin αVβ3 expression in our 3D organotypic model, upon hormonal stimulation, may have resulted from EEC–ESC crosstalk following IL-33–ST2L signalling within the stromal compartment, particularly since rising progesterone levels induce the epithelial PR and ER down-regulation during the secretory stage, permitting progesterone and oestrogen to act only on ESCs (Lessey *et al.*, 1996; Lessey, 1998). However, further experiments would be required to confirm whether these well-known endometrial functional changes are responsible for the lack of induction of integrin αVβ3 in EECs in monoculture that we observed, upon hormonal stimulation, in the current study. In addition, differences were observed with modifications of decidualisation stimulation doses: while ESCs treated with increasing doses that exceeded physiological hormonal and cAMP levels still elicited a transient ST2L up-regulation, continuous stimulatory doses did not (Supplementary Tables SIII and SIV, Fig. S1A). Nonetheless, both of these stimulation protocols (Supplementary Tables SIII and SIV) induced epithelial αVβ3 expression, albeit to a lesser extent (Supplementary Fig. S1C and D) than detected upon treatment with increasing physiological stimulatory doses. Such observations and nuances highlight the significance of appropriate experimental design, and also denote the importance of the interdependent relationship between the timing and level of ovarian hormonal signalling that is a likely requisite in the process of endometrial receptivity.

Limitations of the study

We acknowledge that the simplified functional endometrial organotypic model system presented here does not fully represent all the cellular components and communications that are implicated in the early events leading up to and during the 'window of implantation'. These include glandular epithelial cells that undergo secretory transformation to provide histiotrophic nutrition for the implanting embryo, decidual natural killer cells that have important functions in stromal-immune crosstalk, uterine vascular development, embryo implantation and trophoblast invasion, or vascular components that undergo changes

during the peri-implantation period (Maruyama and Yoshimura, 2008; Cartwright *et al.*, 2010; Weimar *et al.*, 2013; Fraser *et al.*, 2015). However, ESCs are the main cell-type in the uterine microenvironment, and through an initial acute autoinflammatory decidual response, they are pivotal for transforming the uterus into a receptive phenotype by signalling to the overlying epithelium to induce the expression of key receptivity molecules. We have therefore put emphasis on the stromal and luminal epithelial components for the development of our organotypic model system, paracrine interactions of which are central to the generation of endometrial receptivity (Lessey, 1998, 2003; Al-Sabbagh *et al.*, 2011; Salker *et al.*, 2012; Lucas *et al.*, 2016). In addition, our model could benefit from further validation, for example, via photomicrographic verification of accurately representative 3D spatial relationships of the cell-types, through characterisation of EEC and ESC steroid receptor expression upon hormonal stimulation, or by using the iGenomix® (iGenomix UK Ltd, Surrey, UK) Endometrial Receptivity Array genomic tool (Katzorke *et al.*, 2016), for additional confirmation of a receptive endometrial phenotype.

Conclusion

Endometrial cell and molecular signalling errors are widely associated with uterine pathologies ranging from infertility to cancer (Makieva *et al.*, 2018). Any disturbance in decidual transformation of the endometrium, and in turn endometrial receptivity, can cause endometrial functional inadequacy, leading to implantation failure or pregnancy loss resulting from abnormal implantation. Dysregulation of decidualisation and impaired endometrial receptivity have been implicated in infertility, implantation failure, recurrent miscarriage, pre-eclampsia and intrauterine growth restriction (Norwitz, 2006; Strowitzki *et al.*, 2006; Cartwright *et al.*, 2010; Lessey, 2011; Patel and Lessey, 2011; Gellersen and Brosens, 2014; Timeva *et al.*, 2014; Rabaglino *et al.*, 2015; Tan *et al.*, 2015; Conrad *et al.*, 2017). In addition, several gynaecological disorders, including endometriosis, polycystic ovary syndrome, hydrosalpinges and luteal phase defect, are also associated with decreased endometrial receptivity and anomalous expression of endometrial biomarkers (Donaghay and Lessey, 2007). The 3D endometrial organotypic system presented here may therefore facilitate a better understanding of interactions within the uterine microenvironment. Applications could include, for example, assessment of the immunomodulatory and vascular changes that are of critical importance during the secretory stage, implementation of the current model with previously described organoid systems, as well as embryo implantation and trophoblast invasion study protocols (Teklenburg *et al.*, 2010; Fraser *et al.*, 2012; Wang *et al.*, 2012; Wallace *et al.*, 2013; James *et al.*, 2016; Turco *et al.*, 2017). Other potential applications could be for the development of advances in contraceptives or in the investigation of how various drugs (such as those used in infertility or chemotherapeutic treatments) may interfere with endometrial signalling pathways, particularly where human *in vivo* studies are not feasible. The co-culture system developed here, therefore, has the scope to be applied in an extensive range of settings, allowing investigations for the comprehensive understanding of the molecular interactions and cellular consequences within the uterine microenvironment during this early transitory period, in the broad context of several of reproductive, obstetric, and gynaecological pathologies.

Supplementary data

Supplementary data are available at *Human Reproduction Open* online.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

Acknowledgements

The authors thank Professor Hilary Critchley and her team for recruitment of patients and collection of uterine biopsy samples, and we are grateful to all the patients who participated in this research. We also thank Professor Jan Brosens, University of Warwick, for his generous gift of St-T1b cells for this study.

Authors' roles

R.F. conceived the study. R.F. and R.S. performed experiments. R.F. analysed data, prepared the manuscript and was responsible for funding acquisition to provide consumables. C.-J.L. was responsible for funding acquisition to provide salary, space, and equipment for this work to be conducted, and provided critical appraisal of the research. All authors approved the final version of the manuscript.

Funding

This work was supported in part by a Medical Research Council Centre Grant (project reference MR/N022556/1). R.F. was the recipient of a Moray Endowment award and a Barbour Watson Trust award. C.-J.L. is a Royal Society of Edinburgh Personal Research Fellow, funded by the Scottish Government.

Conflict of interest

None declared.

References

Achache H, Revel A. Endometrial receptivity markers, the journey to successful embryo implantation. *Hum Reprod Update* 2006;**12**:731–746.

Al-Sabbagh M, Fusi L, Higham J, Lee Y, Lei K, Hanyaloglu AC, Lam EW, Christian M, Brosens JJ. NADPH oxidase-derived reactive oxygen species mediate decidualization of human endometrial stromal cells in response to cyclic AMP signaling. *Endocrinology* 2011;**152**:730–740.

Apparao KB, Murray MJ, Fritz MA, Meyer WR, Chambers AF, Truong PR, Lessey BA. Osteopontin and its receptor alphavbeta(3) integrin are coexpressed in the human endometrium during the menstrual cycle but regulated differentially. *J Clin Endocrinol Metab* 2001;**86**:4991–5000.

Arnold JT, Kaufman DG, Seppälä M, Lessey BA. Endometrial stromal cells regulate epithelial cell growth *in vitro*: a new co-culture model. *Hum Reprod* 2001;**16**:836–845.

Bentin-Ley U, Horn T, Sjögren A, Sorensen S, Falck Larsen J, Hamberger L. Ultrastructure of human blastocyst-endometrial interactions *in vitro*. *J Reprod Fertil* 2000;**120**:337–350.

Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. *Proc Natl Acad Sci USA* 1986;**83**:2496–2500.

Bläuer M, Heinonen PK, Martikainen PM, Tomás E, Ylikomi T. A novel organotypic culture model for normal human endometrium: regulation of epithelial cell proliferation by estradiol and medroxyprogesterone acetate. *Hum Reprod* 2005;**20**:864–871.

Brar AK, Frank GR, Kessler CA, Cedars MI, Handwerger S. Progesterone-dependent decidualization of the human endometrium is mediated by cAMP. *Endocrine* 1997;**6**:301–307.

Cakmak H, Taylor HS. Implantation failure: molecular mechanisms and clinical treatment. *Hum Reprod Update* 2011;**17**:242–253.

Cartwright JE, Fraser R, Leslie K, Wallace AE, James JL. Remodelling at the maternal-fetal interface: relevance to human pregnancy disorders. *Reproduction* 2010;**140**:803–813.

Chen JC, Erikson DW, Piltonen TT, Meyer MR, Barragan F, McIntire RH, Tamaresis JS, Vo KC, Giudice LC, Irwin JC. Coculturing human endometrial epithelial cells and stromal fibroblasts alters cell-specific gene expression and cytokine production. *Fertil Steril* 2013;**100**:1132–1143.

Conrad KP, Rabaglino MB, Post Uiterweer ED. Emerging role for dysregulated decidualization in the genesis of preeclampsia. *Placenta* 2017;**60**:119–129.

Das A, Mantena SR, Kannan A, Evans DB, Bagchi MK, Bagchi IC. De novo synthesis of estrogen in pregnant uterus is critical for stromal decidualization and angiogenesis. *Proc Natl Acad Sci USA* 2009;**106**:12542–12547.

Denker HW. Implantation: a cell biological paradox. *J Exp Zool* 1993;**266**:541–558.

Donaghay M, Lessey BA. Uterine receptivity: alterations associated with benign gynecological disease. *Semin Reprod Med* 2007;**25**:461–475.

Eckert RL, Katzenellenbogen BS. Human endometrial cells in primary tissue culture: modulation of the progesterone receptor level by natural and synthetic estrogens *in vitro*. *J Clin Endocrinol Metab* 1981;**52**:699–708.

Elnaggar A, Farag AH, Gaber ME, Hafeez MA, Ali MS, Atef AM. AlphaVBeta3 integrin expression within uterine endometrium in unexplained infertility: a prospective cohort study. *BMC Womens Health* 2017;**17**:90.

Fazleabas AT, Kim JJ, Strakova Z. Implantation: embryonic signals and the modulation of the uterine environment—a review. *Placenta* 2004;**25(Suppl A)**:S26–S31.

Fraser R, Whitley GS, Johnstone AP, Host AJ, Sebire NJ, Thilaganathan B, Cartwright JE. Impaired decidual natural killer cell regulation of vascular remodelling in early human pregnancies with high uterine artery resistance. *J Pathol* 2012;**228**:322–332.

Fraser R, Whitley GS, Thilaganathan B, Cartwright JE. Decidual natural killer cells regulate vessel stability: implications for impaired spiral artery remodelling. *J Reprod Immunol* 2015;**110**:54–60.

Freshney RI. Chapter 25: Organotypic culture. *Culture of animal cells: a manual of basic technique, 5th edition*. New York: Wiley, 2005.

Gao JG, Mazella J, Tseng L. Activation of the human IGFBP-1 gene promoter by progestin and relaxin in primary culture of human endometrial stromal cells. *Mol Cell Endocrinol* 1994;**104**:39–46.

Gellersen B, Brosens IA, Brosens JJ. Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. *Semin Reprod Med* 2007;**25**:445–453.

Gellersen B, Brosens J. Cyclic AMP and progesterone receptor cross-talk in human endometrium: a decidualizing affair. *J Endocrinol* 2003;**178**:357–372.

Gellersen B, Brosens JJ. Cyclic decidualization of the human endometrium in reproductive health and failure. *Endocr Rev* 2014;**35**:851–905.

Gellersen B, Kempf R, Telgmann R, DiMattia GE. Nonpituitary human prolactin gene transcription is independent of Pit-1 and differentially controlled in lymphocytes and in endometrial stroma. *Mol Endocrinol* 1994;**8**:356–373.

Giudice LC, Mark SP, Irwin JC. Paracrine actions of insulin-like growth factors and IGF binding protein-1 in non-pregnant human endometrium and at the decidual-trophoblast interface. *J Reprod Immunol* 1998;**39**:133–148.

Idelevich A, Vilella F. Mother and embryo cross-communication. *Genes (Basel)* 2020;**11**:376.

Irwin JC, Kirk D, King RJ, Quigley MM, Gwatkin RB. Hormonal regulation of human endometrial stromal cells in culture: an *in vitro* model for decidualization. *Fertil Steril* 1989;**52**:761–768.

James J, Tun W, Clark A. Quantifying trophoblast migration: *in vitro* approaches to address *in vivo* situations. *Cell Adh Migr* 2016;**10**:77–87.

Kajihara T, Tanaka K, Oguro T, Tochigi H, Prechapanich J, Uchino S, Itakura A, Sućurović S, Murakami K, Brosens JJ *et al.* Androgens modulate the morphological characteristics of human endometrial stromal cells decidualized *in vitro*. *Reprod Sci* 2014;**21**:372–380.

Katzorke N, Vilella F, Ruiz M, Krüssel JS, Simón C. Diagnosis of endometrial-factor infertility: current approaches and new avenues for research. *Geburtshilfe Frauenheilkd* 2016;**76**:699–703.

Kim JJ, Taylor HS, Lu Z, Ladhani O, Hastings JM, Jackson KS, Wu Y, Guo SW, Fazleabas AT. Altered expression of HOXA10 in endometriosis: potential role in decidualization. *Mol Hum Reprod* 2007;**13**:323–332.

Labarta E, Martínez-Conejero JA, Alamá P, Horcajadas JA, Pellicer A, Simón C, Bosch E. Endometrial receptivity is affected in women with high circulating progesterone levels at the end of the follicular phase: a functional genomics analysis. *Hum Reprod* 2011;**26**:1813–1825.

Lessey BA. Endometrial integrins and the establishment of uterine receptivity. *Hum Reprod* 1998;**13**:247–258; discussion 259–261.

Lessey BA. Adhesion molecules and implantation. *J Reprod Immunol* 2002;**55**:101–112.

Lessey BA. Two pathways of progesterone action in the human endometrium: implications for implantation and contraception. *Steroids* 2003;**68**:809–815.

Lessey BA. Assessment of endometrial receptivity. *Fertil Steril* 2011;**96**:522–529.

Lessey BA, Castelbaum AJ. Integrins and implantation in the human. *Rev Endocr Metab Disord* 2002;**3**:107–117.

Lessey BA, Ilesanmi AO, Lessey MA, Riben M, Harris JE, Chwalisz K. Luminal and glandular endometrial epithelium express integrins

differentially throughout the menstrual cycle: implications for implantation, contraception, and infertility. *Am J Reprod Immunol* 1996;**35**: 195–204.

Levin JH, Tonetta SA, Lobo RA. Comparison of norethindrone and medroxyprogesterone acetate with natural progesterone and estradiol in stimulating prolactin production from cultured endometrial stromal cells. *Am J Obstet Gynecol* 1990;**163**:1932–1938.

Liang YX, Liu L, Jin ZY, Liang XH, Fu YS, Gu XW, Yang ZM. The high concentration of progesterone is harmful for endometrial receptivity and decidualization. *Sci Rep* 2018;**8**:712.

Liu X, Ory V, Chapman S, Yuan H, Albanese C, Kallakury B, Timofeeva OA, Nealon C, Dakic A, Simic V *et al.* ROCK inhibitor and feeder cells induce the conditional reprogramming of epithelial cells. *Am J Pathol* 2012;**180**:599–607.

Logan PC, Ponnampalam AP, Steiner M, Mitchell MD. Effect of cyclic AMP and estrogen/progesterone on the transcription of DNA methyltransferases during the decidualization of human endometrial stromal cells. *Mol Hum Reprod* 2013;**19**:302–312.

Lucas ES, Dyer NP, Murakami K, Lee YH, Chan YW, Grimaldi G, Muter J, Brighton PJ, Moore JD, Patel G *et al.* Loss of endometrial plasticity in recurrent pregnancy loss. *Stem Cells* 2016;**34**:346–356.

Ma WG, Song H, Das SK, Paria BC, Dey SK. Estrogen is a critical determinant that specifies the duration of the window of uterine receptivity for implantation. *Proc Natl Acad Sci USA* 2003;**100**:2963–2968.

Makieva S, Giacomini E, Ottolina J, Sanchez AM, Papaleo E, Viganò P. Inside the endometrial cell signaling subway: mind the gap(s). *Int J Mol Sci* 2018;**19**:2477.

Maruyama T, Yoshimura Y. Molecular and cellular mechanisms for differentiation and regeneration of the uterine endometrium. *Endocr J* 2008;**55**:795–810.

Michalski SA, Chadchan SB, Jungheim ES, Kommagani R. Isolation of human endometrial stromal cells for *in vitro* decidualization. *J Vis Exp* 2018;**139**:57684.

Norwitz ER. Defective implantation and placentation: laying the blueprint for pregnancy complications. *Reprod Biomed Online* 2006;**13**: 591–599.

Norwitz ER, Schust DJ, Fisher SJ. Implantation and the survival of early pregnancy. *N Engl J Med* 2001;**345**:1400–1408.

Okada H, Tsuzuki T, Murata H. Decidualization of the human endometrium. *Reprod Med Biol* 2018;**17**:220–227.

Palechor-Ceron N, Suprynowicz FA, Upadhyay G, Dakic A, Minas T, Simic V, Johnson M, Albanese C, Schlegel R, Liu X. Radiation induces diffusible feeder cell factor(s) that cooperate with ROCK inhibitor to conditionally reprogram and immortalize epithelial cells. *Am J Pathol* 2013;**183**:1862–1870.

Patel B, Elguero S, Thakore S, Dahoud W, Bedaiwy M, Mesiano S. Role of nuclear progesterone receptor isoforms in uterine pathophysiology. *Hum Reprod Update* 2015;**21**:155–173.

Patel BG, Lessey BA. Clinical assessment and management of the endometrium in recurrent early pregnancy loss. *Semin Reprod Med* 2011;**29**:491–506.

Paulson RJ. Hormonal induction of endometrial receptivity. *Fertil Steril* 2011;**96**:530–535.

Rabaglino MB, Post Uiterweer ED, Jeyabalan A, Hogge WA, Conrad KP. Bioinformatics approach reveals evidence for impaired endometrial maturation before and during early pregnancy in women who developed preeclampsia. *Hypertension* 2015;**65**:421–429.

Rai V, Hopkisson J, Kennedy S, Bergqvist A, Barlow DH, Mardon HJ. Integrins alpha 3 and alpha 6 are differentially expressed in endometrium and endometriosis. *J Pathol* 1996;**180**:181–187.

Salamonsen LA, Nie G, Hannan NJ, Dimitriadis E. Society for Reproductive Biology Founders' Lecture 2009. Preparing fertile soil: the importance of endometrial receptivity. *Reprod Fertil Dev* 2009;**21**:923–934.

Salker MS, Nautiyal J, Steel JH, Webster Z, Sucurovic S, Nicou M, Singh Y, Lucas ES, Murakami K, Chan YW *et al.* Disordered IL-33/ST2 activation in decidualizing stromal cells prolongs uterine receptivity in women with recurrent pregnancy loss. *PLoS One* 2012;**7**: e52252.

Samalecos A, Reimann K, Wittmann S, Schulte HM, Brosens JJ, Bamberger AM, Gellersen B. Characterization of a novel telomerase-immortalized human endometrial stromal cell line, St-T1b. *Reprod Biol Endocrinol* 2009;**7**:76.

Simian M, Bissell MJ. Organoids: a historical perspective of thinking in three dimensions. *J Cell Biol* 2017;**216**:31–40.

Strowitzki T, Germeyer A, Popovici R, von Wolff M. The human endometrium as a fertility-determining factor. *Hum Reprod Update* 2006;**12**:617–630.

Suprynowicz FA, Upadhyay G, Krawczyk E, Kramer SC, Hebert JD, Liu X, Yuan H, Cheluvaraju C, Clapp PW, Boucher RC Jr *et al.* Conditionally reprogrammed cells represent a stem-like state of adult epithelial cells. *Proc Natl Acad Sci USA* 2012;**109**:20035–20040.

Tamura I, Jozaki K, Sato S, Shirafuta Y, Shinagawa M, Maekawa R, Taketani T, Asada H, Tamura H, Sugino N. The distal upstream region of insulin-like growth factor-binding protein-1 enhances its expression in endometrial stromal cells during decidualization. *J Biol Chem* 2018;**293**:5270–5280.

Tan SY, Hang F, Purvarshi G, Li MQ, Meng DH, Huang LL. Decreased endometrial vascularity and receptivity in unexplained recurrent miscarriage patients during midluteal and early pregnancy phases. *Taiwan J Obstet Gynecol* 2015;**54**:522–526.

Tarrade A, Goffin F, Munaut C, Lai-Kuen R, Tricottet V, Foidart JM, Vidaud M, Frankenne F, Evain-Brion D. Effect of matrigel on human extravillous trophoblasts differentiation: modulation of protease pattern gene expression. *Biol Reprod* 2002;**67**:1628–1637.

Teklenburg G, Salker M, Molokhia M, Lavery S, Trew G, Aojanepong T, Mardon HJ, Lokugamage AU, Rai R, Landles C *et al.* Natural selection of human embryos: decidualizing endometrial stromal cells serve as sensors of embryo quality upon implantation. *PLoS One* 2010;**5**:e10258.

Timeva T, Shterev A, Kyurkchiev S. Recurrent implantation failure: the role of the endometrium. *J Reprod Infertil* 2014;**15**:173–183.

Turco MY, Gardner L, Hughes J, Cindrova-Davies T, Gomez MJ, Farrell L, Hollinshead M, Marsh SGE, Brosens JJ, Critchley HO *et al.* Long-term, hormone-responsive organoid cultures of human endometrium in a chemically defined medium. *Nat Cell Biol* 2017; **19**:568–577.

Vinketova K, Mourdjeva M, Oreshkova T. Human decidual stromal cells as a component of the implantation niche and a modulator of maternal immunity. *J Pregnancy* 2016;**2016**:8689436.

Wallace AE, Host AJ, Whitley GS, Cartwright JE. Decidual natural killer cell interactions with trophoblasts are impaired in pregnancies at increased risk of preeclampsia. *Am J Pathol* 2013;**183**:1853–1861.

Wang H, Pilla F, Anderson S, Martínez-Escribano S, Herrer I, Moreno-Moya JM, Musti S, Bocca S, Oehninger S, Horcajadas JA. A novel model of human implantation: 3D endometrium-like culture system to study attachment of human trophoblast (Jar) cell spheroids. *Mol Hum Reprod* 2012;**18**:33–43.

Weimar CH, Post Uiterweer ED, Teklenburg G, Heijnen CJ, Macklon NS. Reprint of: *in-vitro* model systems for the study of human embryo-endometrium interactions. *Reprod Biomed Online* 2013;**27**:673–688.

Yu J, Berga SL, Johnston-MacAnanny EB, Sidell N, Bagchi IC, Bagchi MK, Taylor RN. Endometrial stromal decidualization responds reversibly to hormone stimulation and withdrawal. *Endocrinology* 2016;**157**:2432–2446.