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ORIGINAL RESEARCH

Critical evaluation of human endometrial explants as an *ex vivo* model system: a molecular approach

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ABSTRACT: The human endometrium is unique among adult tissues. Its functions are modulated by numerous hormones and mediators. The aim of this study was to evaluate the suitability of human endometrial explants for studying functional effects of chemicals and drugs on gene expression biomarkers. Endometrial tissues were obtained by aspiration curettage and cultivated for up to 24 h. Relative mRNA concentrations were determined by reverse transcription quantitative real-time PCR. Viability was assessed by light microscopy, lactate dehydrogenase assay and scanning electron microscopy. It was acceptable after 6 h of culture but reduced after 24 h. Culture-induced alterations of mRNA levels were found for progesterone receptor, estrogen receptor$_\alpha$, leukemia inhibitory factor and cyclooxygenase-2 in tissues from all cycle stages. The suitability of the model to detect chemical effects was demonstrated by the down-regulation of cyclooxygenase-2 mRNA by chlormadinone acetate in proliferative and secretory endometrium. The model is mainly restricted by interindividual variations and varying tissue quality. An advantage is the preservation of tissue composition. We conclude that human endometrial explants are a complex model due to limited viability, difficult standardization and intrinsic alterations during culture. Experiments with this model should be performed over a limited time period under strictly controlled conditions.

Key words: chlormadinone acetate / endometrium / gene expression biomarkers / RT-qPCR / tissue culture

Introduction

The human endometrium is an unique adult tissue due to the complex dynamic changes it undergoes during each menstrual cycle (Jabbour *et al.*, 2006). It hosts the embryo implantation process and is involved in various dysfunctions and pathologies of the female reproductive tract (e.g. infertility, dysmenorrhea, endometriosis). The complex morphological structure of the endometrium consists mainly of stromal and epithelial cells, but also contains endothelial and immune cells.

Endometrial function is mainly controlled by endocrine action of 17β-estradiol and progesterone, and on subordinate levels by many local mediators (e.g. growth factors, cytokines). Disorders of the menstrual cycle (e.g. dysmenorrhea) may be associated with alterations of endometrial function (Jabbour *et al.*, 2006). The endometrium has also been recognized as a fertility-determinating factor in the process of embryo implantation (Strowitzki *et al.*, 2006). Endometrial functions can be affected by drugs (e.g. contraceptives) or chemical exposure.

A broad spectrum of cell culture-based methods is available for studying the multifaceted functions of the human endometrium (Bremer *et al.*, 2007). Human endometrial stromal and epithelial cells can be cultured as individual primary cell cultures or as co-cultures (Classen-Linke *et al.*, 1997); in co-cultures interactions of epithelial and stromal cells can be studied (Arnold *et al.*, 2001; Lalitkumar *et al.*, 2007). Due to their functional importance, primary human endometrial endothelial cells are also a relevant research model. In addition to primary cell cultures, a variety of permanent human endometrial epithelial cell lines (e.g. Ishikawa, ECC-1) are well established (Mo *et al.*, 2006; Boehme *et al.*, 2009; Naciff *et al.*, 2010). Human endometrial stromal cells are available as immortalized cell lines (e.g. Yale human endometrial stromal cells; Krikun *et al.*, 2004). A major disadvantage of tumor and immortal cell lines is that they often have lost important characteristics of their progenitors.

The closest approach to *in vivo* conditions, however, are cultures of human endometrial explants, where tissue composition and

communication between cells are maintained (e.g. paracrine interactions between stromal and epithelial cells) (Fasciani *et al.*, 2003; Stavreus-Evers *et al.*, 2003). Besides epithelial and stromal cells, other functionally important cell types (e.g. endothelial cells, macrophages) are preserved. Explants cultures have been used for studying endometrial physiology and pathophysiology (Dudley *et al.*, 1992; Cornet *et al.*, 2002; Stavreus-Evers *et al.*, 2003; Punyadeera *et al.*, 2004; Bellehumeur *et al.*, 2005; Vassilev *et al.*, 2005) as well as for pharmacological and toxicological investigations (Pitt *et al.*, 2001; Catalano *et al.*, 2003; Jain *et al.*, 2005; Kim *et al.*, 2005).

The use of human endometrial explants cultures as a model system presents some experimental problems and limitations. One limitation is the availability of endometrium. Endometrial biopsies in published studies mainly were obtained from surgeries for various benign indications. For a minor portion of studies, volunteers were recruited under better definable conditions. Usually the intake of hormones 3 months prior to sampling must be excluded. A critical aspect is the time point of tissue sampling in the menstrual cycle, which must be assured by appropriate methods. The limited explants viability, sample quality, the hormonal milieu *ex vivo* and oxygenation are additional experimental problems. Those restrictions of human endometrial explants cultures, e.g. the viability problem, were addressed only in a few publications (Dudley *et al.*, 1992; Stavreus-Evers *et al.*, 2003).

The aim of this study was to critically evaluate the restrictions and applicability domains of human endometrial explants available in a clinical setting for studying tissue-specific effects of hormonally active compounds. Pre-selected endometrial gene expression biomarkers were analyzed as functional endpoints by the highly sensitive RT-qPCR method due to the low available tissue amounts. Tissue viability was assessed at various time points and alterations of target gene expression were explored under the influence of tissue preparation, culture conditions and exogenous test compounds.

Materials and Methods

Tissue collection

Endometrial tissues were obtained by aspiration curettage using the Pipelle endometrial biopsy instrument (Pipelle De Cornier, Laboratoire C.C.D., Paris, France) from 65 premenopausal women undergoing surgery for benign gynecological disorders. The study was approved by the Ethical Committee of the University of Freiburg (ethical votes no. 154/06 and 300/06) and patients gave their written informed consent. All patients had no hormonal treatment for 3 months. The tissue amounts obtained were 210 ± 146 mg (33–762 mg). The mean age of the patients was 35.5 ± 7.1 years (19–47 years). The biopsies were transferred immediately to the laboratory in the Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Taufkirchen, Germany). At least 30 mg of tissue was needed for one complete explants culture experiment. Another aliquot of the biopsies was stored immediately after sampling in RNAlater (Qiagen, Hilden, Germany) at 4°C for investigation of basal mRNA levels of target and reference genes in non-cultured samples. Approximately 70 mg of the biopsies were preserved in 4% formaldehyde (VWR International, Darmstadt, Germany) for histological examination when the sample amount was >200 mg. Patients' blood was collected for the determination of LH, FSH, 17β-estradiol and progesterone, and the menstrual cycle history was recorded. Since the date of surgery was dependent on clinical requirements, the tissues studied were from different stages of the menstrual cycle.

Endometrial explants culture

Preparation of explants was performed under sterile conditions under a laminar flow (Antares 48, BIOHIT, Rosbach v.d.H., Germany). The endometrial biopsies were separated from the transport medium by sieving through Shandon tissue specimen bags (Thermo Electron, Karlsruhe, Germany), rinsed in Dulbecco's phosphate buffered saline (D-PBS; GIBCO/Invitrogen, Karlsruhe, Germany) and cleared of blood and mucus. Subsequently, the tissue was chopped into pieces of 1–2 mm/side and pre-incubated in DMEM/Ham's F-12 medium (Sigma-Aldrich; Art. No. D6434, without phenol red, endotoxin tested) containing 2 mM L-glutamine (GIBCO/Invitrogen) for 1 h.

For the main incubation the explants (3–5 pieces, total wet weight ≥5 mg per well) were placed into a 24-well Costar plate (Vitaris, Baar, Switzerland) on Millicell-PCF inserts (12 μm; Millipore, Eschborn, Germany) and cultivated in 1 ml fresh DMEM/Ham's F-12 medium (with 2 mM L-glutamine, without phenol red) for up to 24 h. In one experiment, explants were treated with the synthetic progestin chlormadinone acetate (CMA, 10^{-6} M, CAS-No. 302-22-7; Grünenthal-International, Aachen, Germany) and human recombinant interleukin-1β (IL-1β, 1 ng/ml; Sigma-Aldrich, product no. I9401) using 0.1% ethanol as vehicle. CMA treatment started at the beginning of the main incubation period and IL-1β was added after 1 h for further 5 h. In a second experiment, explants were treated with the synthetic estrogen diethylstilbestrol (DES; 10^{-6} M, CAS No. 56-53-1; Sigma-Aldrich) for 8 h. Incubation times were selected after investigation of time courses in respective pilot experiments. Controls were incubated with vehicle only (0.1% ethanol). Due to the low tissue amounts usually no replicates were run. In some experiments without synthetic test compounds, the medium was supplemented with 17β-estradiol (10^{-9} M) and progesterone (10^{-7} M; both from Sigma-Aldrich) or fetal bovine serum (FBS Gold; EU approved; PAA Laboratories, Pasching, Austria). Uniform hormonal conditions were applied in these experiments since the results from menstrual cycle dating (hormone values, histological data) were not available at the time of explants culture experiments.

All incubations were performed in a humidified atmosphere at 37°C with 5% CO_2 in a CO_2 incubator (Heracell 150, Thermo Electron LED, Langenselbold, Germany). After termination of the incubation period, the pooled explants of each well were preserved separately in RNA later at 4°C for approximately 24 h, subsequently shock frozen in liquid nitrogen and stored at −80°C until RNA isolation.

Lactate dehydrogenase assay

The lactate dehydrogenase (LDH) assay was used as a viability assay, which is applicable in tissue culture (Benford and Hubbard, 1987). Leakage of LDH into the culture medium is indicative for cell membrane damage. Extracellular LDH was measured in the culture media of explants cultures after centrifugation. For the measurement of intracellular LDH, the snap-frozen explants were homogenized by bead milling (microdismembrator II; Braun, Melsungen, Germany) in a 50 mM Tris buffer of pH 7.4 (Trizma, Sigma-Aldrich) containing 50 mM potassium chloride (VWR International), and the LDH activity was determined in the supernatants after centrifugation. For LDH measurements, 675 μl of substrate solution and 25 μl of a 15 mM NADH solution (disodium salt, grade II; Roche Diagnostics; in 1% sodium hydrogen carbonate) were mixed with 300 μl of supernatant in a 24-well plate. The substrate solution contained 44.7 mM dipotassium hydrogen phosphate (Roth, Karlsruhe, Germany), 7.35 mM potassium dihydrogen phosphate (VWR International) and 0.625 mM sodium pyruvate (Sigma-Aldrich). The absorbance was measured

at 340 nm on a μQuant spectrophotometer and evaluated with Gen5 data analysis software (BioTek Instruments, Bad Friedrichshall, Germany). Viability was calculated from the quotient of the intracellular LDH activity and the total LDH activity (extracellular plus intracellular LDH).

Histological examination

Approximately 70 mg of endometrial tissue were fixed in 4% formaldehyde, embedded in paraffin, cut into 5 μm slices and stained with hematoxylin and eosin. Viability and menstrual cycle date were assessed by an experienced pathologist following established microscopic criteria (Blaustein, 1980; Johannisson *et al.*, 1987).

Scanning electron microscopy

Endometrial explants (14–36 mg) were fixed in 3% glutaraldehyde in a 0.2 M sodium cacodylate buffer, pH 7.4 (Merck Eurolab, Darmstadt, Germany). After dehydration in graded ethanol (70–100%), samples were dried in a critical-point dryer (Polaron, Watford, UK) mounted on stubs and coated with gold palladium in a cool sputter coater (Fisons Instruments Uckfield, UK). The specimens were examined in a scanning electron microscope DSM 960 (Zeiss, Oberkochen, Germany).

RNA extraction and cDNA preparation

Total RNA was extracted from disrupted and homogenized frozen tissue specimens by using RNeasy® mini-columns with on-column-DNase digestion (Qiagen). Homogenization was performed by bead milling (microdismembrator II; Braun). For total RNA quantification, absorbance at 260 nm was measured in a NanoDrop spectrophotometer (PeqLab, Erlangen, Germany). Concentrations of RNA templates were >100 ng/μl. The quality of the total RNA was controlled by *RNA 6000 Nano Assays* on a Bioanalyzer 2100 (Agilent Technologies, Böblingen, Germany) and assessed as acceptable if the RNA integrity number (RIN) was >5 (Fleige and Pfaffl, 2006).

cDNA synthesis from total RNA (1 μg) was carried out in a reaction volume of 20 μl containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 5 mM dithiothreitol, 0.5 mM deoxynucleoside triphosphate mix, 200 units SuperScript™ III reverse transcriptase (all from Invitrogen) and 1 μl random primers (hexanucleotide mix, 10×; Roche Diagnostics). Initially, RNA was denatured at 65°C for 5 min, then the reaction mixture was added and reverse transcription was performed at 50°C for 50 min. The reaction was stopped by denaturing the enzyme at 80°C for 15 min. The cDNA was stored at ≤ -20°C.

Reverse transcription quantitative real-time PCR

qPCR runs were performed on a LightCycler® 480 instrument in duplicates on LightCycler® 480 plates with 384 wells (Roche Diagnostics). A LightCycler® 480 Probes Master Mix (Roche Diagnostics) containing a FastStart Taq DNA polymerase for hot-start PCR was used. In all experiments, cDNA derived from 40 ng total RNA was used. All qPCR assays were designed from the Universal Probe Library (UPL, Human; Roche Diagnostics) for the genes of interest listed in Table I. Primer and FAM-labeled probe concentrations were 200 and 100 nM, respectively. After an initial activation (5 min, 95°C) 45 cycles were run with denaturation for 10 s at 95°C followed by primer annealing and elongation for 30 s at 60°C. Fluorescence signals were detected at 483–533 nm. For assessing the absence of genomic DNA and contaminating cDNA no-template and no-reverse transcription controls were run for each gene. Evaluation was performed by calibrator-normalized relative quantification with efficiency correction. Calculation of the qPCR results was based on external standard curves elaborated with DNA standards, which had been generated by conventional PCR. A pool of cDNA from 18 patient samples (6 proliferative phase, 12 secretory phase) was used as calibrator, which was included in each qPCR run. The main purpose of the calibrator was its application for the normalization of final qPCR results. Therefore, the target/reference ratios of all samples were divided by the target/reference ratio of the calibrator. The calibrator was used to normalize all samples within one run and also provided a constant calibration point between several qPCR runs. The calculation of the calibrator-normalized ratio does not require a standard curve in each qPCR run. In our experiments, aliquots of the pooled calibrator were integrated over a 3-years period in each qPCR run analyzing endometrial explants. Data analysis was performed with LightCycler® 480 Relative Quantification Software (version 1.5; Roche Diagnostics). Results of qPCR runs are given as normalized ratios. G6PDH, ALAS1, PGK1 and β2M were identified as suitable reference genes from an initial panel of eight reference genes (Table I) by using a systematic, software-based approach (NormFinder, geNORM; Vandesompele *et al.*, 2002; Andersen *et al.*, 2004).

Statistics

Significance testing of differences of paired observations was done using the Wilcoxon signed rank test. *P*-values were not adjusted for multiple testing, results are reported as being significant when the unadjusted *P*-value is <0.05.

Results

In a 3 years period, a total of approximately 200 endometrium specimens were obtained by aspiration curettage (Pipelle) from premenopausal women in different stages of the menstrual cycle. Explants from 65 patients were used for the experiments reported in this study, the remaining samples were used for other trials or training experiments. Approximately 20% of all samples were discarded for too low tissue amount or poor quality. The day of surgery was dependent from clinical demands and could not be determined according to our research needs. A major part of the samples was contaminated by blood and mucus or macroscopically of heterogenous appearance. About 30 mg tissue was needed for one test incubation. At least 70 mg were necessary for histological examination. Cycle history and serum hormone levels (LH, FSH, progesterone and 17β-estradiol) were available for most of the donors. Sonographic data and histological examination was available only for approximately 30% of the donors due to organisational reasons (e.g. outpatient treatment).

Assessment of viability by light microscopy and LDH assay

Histological examination by light microscopy revealed that cultured human endometrial explants from all cycle stages have a limited viability. Explants from both the proliferative and secretory phases displayed only few morphological alterations after 6 h of culture. The stromal architecture appeared slightly rarified with some aggregations of stromal cells. The lumina of the glands were widened. No histological signs of apoptosis and necrosis were found.

After 24 h of culture a significant degeneration of tissue integrity was observed. Fragmentation of glands became evident. Epithelial and mesenchymal compartments were located apart and crevices between basal membranes and stroma appeared. Supplementation of the medium with 10% FBS did not improve tissue morphology (Fig. 1).

Table I qPCR assay specifications for the genes under investigation.

Gene	Gene symbol	Name	Reference Sequence	Primers[a]	UPL probe	Amplicon (bp)	Length of intron spanned	PCR efficiency	Limit of detection (C_q)
Target genes									
PR	PGR	Progesterone receptor	NM_000926	s: 5′-TTTAAGAGGGCAATGGAAGG-3′ as: 5′-CGGATTTTATCAACGATGCAG-3′	#11	74	34127 nt	1.88	34.3
ER_α	ESR1	Estrogen receptor α	NM_000125	s: 5′-TTACTGACCAACCTGGCAGA-3′ as: 5′-ATCATGGAGGGTCAAATCCA-3′	#24	90	67143 nt	1.84	37.9
LIF	LIF	Leukemia inhibitory factor	NM_002309	s: 5′-TGCCAATGCCCTCTTTATTC-3′ as: 5′-GTCCAGGTTGTTGGGGAAC-3′	#26	64	693 nt	1.95	35.5
COX-2	PTGS2	Cyclooxygenase-2	NM_000963	s: 5′-GCTTTATGCTGAAGCCCTATGA-3′ as: 5′-TCCAACTCTGCAGACATTTCC-3′	#2	70	481 nt	1.96	35.3
Reference genes									
ALAS1	ALAS1	Delta-aminolevulinate-synthase 1	NM_199166	s: 5′-GCCTCTGCAGTCCTCAGC-3′ as: 5′-AACAACACTCTCCATGTTCAGG-3′	#36	69	951 nt	1.84	35.9
G6PDH	G6PD	Glucose-6-phoshate dehydrogenase	NM_000402	s: 5′-AACAGAGTGAGCCCTTCTTCA-3′ as: 5′-GGAGGCTGCATCATCGTACT-3′	#5	107	548 nt	1.92	33.3
β2M	B2M	β2-microglobulin	AB021288	s: 5′-TTCTGGCCTGGAGGCTATC-3′ as: 5′-TCAGGAAATTTGACTTTCCATTC-3′	#42	86	3807 nt	1.80	34.0
PGK1	PGK1	Phosphoglycerate kinase 1	NM_000291	s: 5′-TGCAAAGGCCTTGGAGAG-3′ as: 5′-TGGATCTTGTCTGCAACTTTAGC-3′	#72	75	4663 nt	1.89	38.2
HPRT	HPRT1	Hypoxanthine phosphoribosyl-transferase 1	L29382	s: 5′-TGACCTTGATTTATTTTGCATACC-3′ as: 5′-CGAGCAAGACGTTCAGTCCT-3′	#73	102	1712 nt	n.d.	n.d.
PBGD	HMBS	Porphobilinogen deaminase	X04808	s: 5′-CGCATCTGGAGTTCAGGAGTA-3′ as: 5′-TGCTAGGATGATGGCACTGA-3′	#18	92	1147 nt	n.d	n.d.
RPII	POLR2A	Polymerase (RNA) II (DNA directed) polypeptide A	NM_000937	s: 5′-GCAAATTCACCAAGAGAGACG-3′ as: 5′-CACGTCGACAGGAACATCAG-3′	#1	73	1145 nt	n.d.	n.d.
ACTB	ACTB	Beta-Actin	NM_001101	s: 5′-CCAACCGCGAGAAGATGA-3′ as: 5′-CCAGAGGCGTACAGGGATAG-3′	#64	97	438 nt	1.90	36.4

n.d., not determined.
[a]Primers for all assays were intron-spanning.

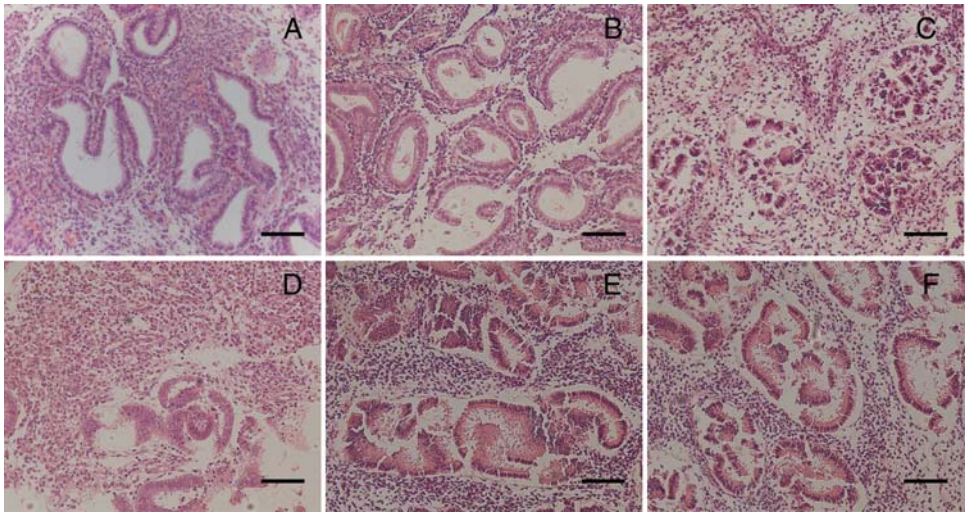

Figure 1 Morphology of non-cultured and cultured human endometrium explants. Representative tissues from three different individuals are presented. In A–C endometrial sections from the same patient (secretory phase) are shown: non-cultured (**A**), after 6 h of culture (**B**), and after 24 h of culture (**C**). (**D**) Endometrium of a different patient (proliferative phase) after 24 h of culture. In (**E**) and (**F**) endometrial explants from a third patient (secretory phase) are shown after 24 h of culture without (E) and with (F) 10% FBS. Fragmentation of glands, crevices between basal membranes and stroma, as well as separation of epithelial and mesenchymal compartments was observed in all explants cultivated over 24 h. Scale bars indicate 125 μm.

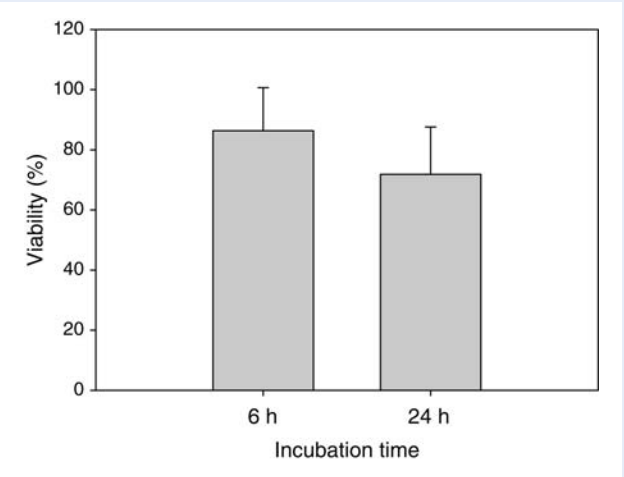

Figure 2 Viability assessment of human endometrial explant cultures by LDH assay. The percentage of viable cells after 6 and 24 h of culture is given as mean ± SD ($n = 6$).

Mean viabilities ± SD assessed by LDH assay were 86.3 ± 14.3% after 6 h and 71.8 ± 15.8% after 24 h of culture (Fig. 2).

Scanning electron microscopy

Tissues from the proliferative and the secretory phases were also investigated by SEM in non-cultured tissues as well as after 6 and 24 h of culture (Figs 3 and 4). The non-cultured specimens displayed typical features of the respective cycle phase and glandular openings were free from cellular detritus. In cultured explants signs of disintegration increased with culture duration. Six hours after culture beginning disintegration of glands was observed in secretory endometrium and dying cells of irregular shape leaving the tissue were found in both cycle phases. Dying epithelial cells are characterized by loosing their apical and basal orientation and becoming rounded. They further have an irregular shape, and partly display holes in the membrane. Finally they fall off the tissue. In Fig. 3D a cell with holes in the membrane is shown. Besides it, a similar shaped cell is leaving the tissue. Similar shaped cells or cells of irregular shape falling off the tissue are also seen in Figs 3C, 4D and E. More distinct signs of degeneration, e.g. fissured gland openings filled with detached cells or increased disintegration of endometrium, were present after 24 h of culture. In secretory endometrium, tissue damage was more pronounced and disappearance of epithelial cells was obvious. Ciliated cells, however, still looked intact even after 24 h of culture, in both proliferative and secretory endometrium.

Alterations of target gene mRNA expression after explants culture

In an experiment with 12 endometrial explants from different cycle phases mRNA expression of four target genes was investigated by RT-qPCR in non-cultured biopsies and after 6 h of culture in the presence of 17β-estradiol (10^{-9} M) and progesterone (10^{-7} M) but in the absence of any test chemicals. The mRNA levels of progesterone receptor (PR) and estrogen receptor (ER_α) were significantly reduced and those of leukemia inhibitory factor (LIF) and cyclooxygenase-2 (COX-2) significantly increased after that time compared with non-cultured endometrium (Fig. 5). In Fig. 5A line plots of normalized mRNA ratios for the different targets are presented for each individual. Samples from the proliferative phase are assigned by

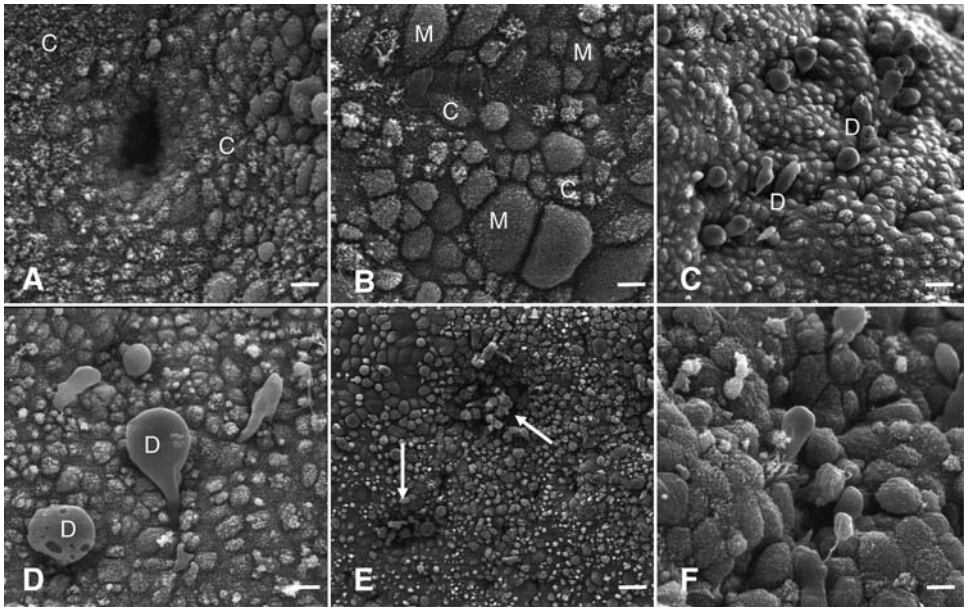

Figure 3 SEM micrographs of non-cultured and cultured proliferative endometrium. Biopsies were obtained at Day 11 of the menstrual cycle. Explants were cultured in DMEM/Ham F12-medium with 10^{-7} M progesterone and 10^{-9} M 17β-estradiol. (**A**) Non-cultured: the endometrium looks normal for the proliferative phase. Glandular opening is free from cellular detritus, ciliated cells are arranged around gland openings (1000×). (**B**) Non-cultured: ciliated cells (*C*) as well as microvilli covered cells (*M*) are evident (2000×). (**C**) 6 h culture: dying cells (*D*) are leaving the tissue (1000×). (**D**) 6 h culture: dying cells (*D*) leaving the tissue, partially displaying destroyed membranes (2000×). (**E**) 24 h culture: fissured gland openings (arrows), filled with detached cells (500×). (**F**) Increased disintegration of endometrium after 24 h of culture (2000×). Scale bars indicate 5 μm in B, D and F, 10 μm in A and C, and 20 μm in F.

open circles, such from the secretory phase by filled circles. Dots on the left side represent mRNA ratios in non-cultured samples, dots on the right side represent mRNA ratios after 6 h of incubation. In Fig. 5B alterations of mRNA levels for the different target genes during culture are summarized as dotplot. Fold change <1 indicates down-regulation, >1 up-regulation of the respective gene of interest during culture. Similar results were obtained for explants incubated in the absence of 17β-estradiol and progesterone (not shown).

Treatment of explants with exogenous test compounds

In a first experiment endometrial explants were treated with the synthetic progestin CMA in order to study its glucocorticoid partial action on COX-2 mRNA expression. One hour after the beginning of CMA treatment, a part of the tissues were additionally challenged by IL-1β (1 ng/ml) and cultivated for further 5 h. COX-2 mRNA was significantly down-regulated by CMA (10^{-6} M) after 6 h of culture in untreated as well as in IL-1β stimulated samples from both cycle phases (Fig. 6). The interindividual variation of normalized ratios was high.

A second experiment with DES as test substance in combination with PR mRNA as biomarker for estrogenic effects is shown in Fig. 7. DES was used within our ReProTect project (an Integrated Project funded by the European Union) as reference compound for strong estrogens in approximately 20 different *in vitro* assays covering various stages of the reproductive cycle. No significant effects on PR mRNA levels were found after treatment with DES (10^{-6} M) compared with untreated controls in this experimental setting with explants from both cycle phases.

Discussion

In this study we addressed critical aspects of analyzing gene expression biomarkers in endometrial explants cultures as a model for toxicological and pharmacological research. We identified viability, intrinsic variability and alterations of gene expression during culture as limitations of this model. Although endometrial explants cultures are widely applied, studies addressing these constraints are rare (Dudley *et al.*, 1992; Stavreus-Evers *et al.*, 2003).

In our study, tissue viability was assessed by morphological evaluation, LDH assay, and SEM after 6 and 24 h of culture. Taken together over all findings, viability was acceptable after 6 h but reduced after 24 h of culture. The (non-significant) loss of viability observed by LDH assay after 6 h is supposed to be mainly due to tissue damage caused by the preparation procedure, since the tissue morphology assessed by light microscopy was preserved. By SEM, however, dying cells evading from the tissue were already detectable after 6 h of culture in tissues from the proliferative as well as the secretory phases. Degeneration was significantly more manifest after 24 h of culture. Ciliated cells still looked fine after 24 h indicating that they endure cultivation. Viability was not improved by supplementation of the culture medium with 10% FBS. Therefore, we performed our experiments with test compounds generally under serum-free conditions.

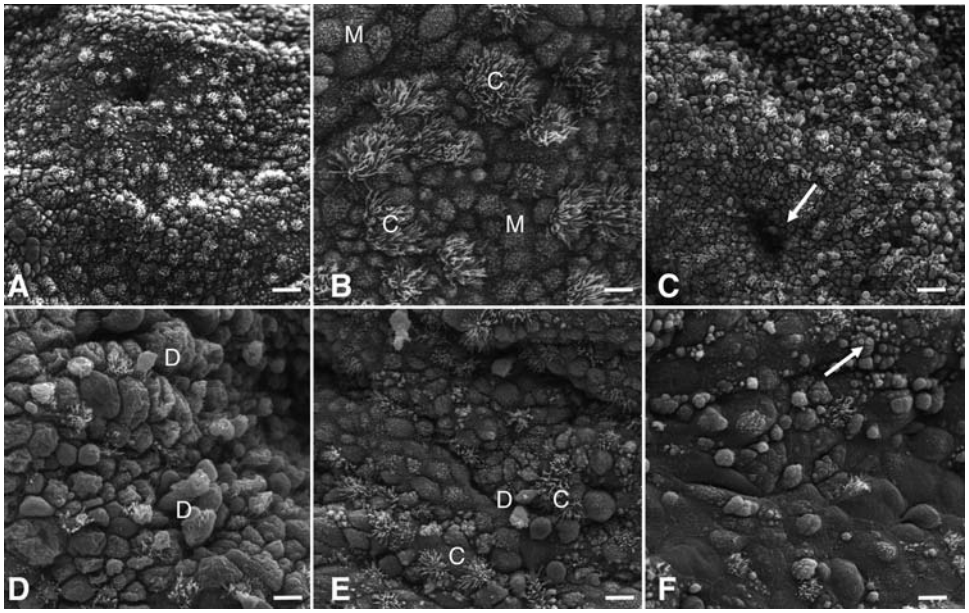

Figure 4 SEM micrographs of non-cultured and cultured secretory endometrium. Biopsies were sampled in the early secretory phase. Explants were cultured in DMEM/Ham F12-medium with 10^{-7} M progesterone and 10^{-9} M 17β-estradiol. (**A**) Non-cultured: Normal appearance of secretory endometrium, approximately 5 d after ovulation (500×). (**B**) Non-cultured: Ciliated cells (*C*) as well as microvilli covered cells (*M*) are evident (2000×). (**C**) 6 h culture: Beginning disintegration of glands (arrow; 500×). (**D**) 6 h culture: dying cells (*D*) of irregular shape are leaving the tissue (1000×). (**E**) 24 h culture: dying cells are leaving the tissue. Ciliated cells seem to be still intact (1000×). (**F**) After 24 h culture: disappearence of epithelial cells, the remaining cells of uneven size form a carpet (arrow; 1000×). Scale bars indicate 5 μm in B, 10 μm in D, E and F and 20 μm in A and C.

Our viability results are comparable to those of another study (Stavreus-Evers *et al.*, 2003), which described a good preservation of endometrial tissues after 8 h of culture. Taken together, these findings demonstrate that cultivation of endometrial explants up to 8 h is uncritical, but for longer cultivation periods sufficient viability data should be provided. The observed rapid loss of viability *ex vivo* might be a consequence of the detachment of the endometrium from the uterus. It has been reported by others, however, that some functional characteristics might be retained by endometrial explants for several days despite morphological degeneration (Dudley *et al.*, 1992; Stavreus-Evers *et al.*, 2003; Fogle *et al.*, 2010). In any case, we recommend to monitor tissue viability in endometrial explants studies and to carefully validate observed effects by time-matched control experiments.

Another major complication in studies with endometrial explants sampled in a clinical setting is interindividual variation due to cycle phase, age, intrauterine disorders and exposure to exogenous hormones. Some of these problems can be avoided, e.g. by exclusion of patients under hormone treatment. Others, like the periodical changes in endometrial function are difficult to control. In this context patient selection is a major problem, since sampling of endometrium in a clinical setting usually cannot be timed according to research requirements and therefore not to be adjusted to distinct cycle stages. Moreover, the results from menstrual cycle dating (hormone values, histological findings) are usually not available at the time of explants culture. In our study and most published investigations endometrial biopsies were obtained from surgeries for benign indications. Drawbacks of this approach are the higher age of patients, sampling at variable menstrual cycle stages and underlying pathologies. Alternatively, volunteers can be recruited for sampling at a pre-selected time point of the menstrual cycle (Stavreus-Evers *et al.*, 2003); however, for such an approach only a limited number of subjects can be enrolled.

The quality and quantity of tissues is a further problem in investigating endometrial explants. The original localization of the biopsy in the uterine cavity is unknown and not reconstitutable in culture. Further, the specimens contain varying portions of mucus and blood, which must be removed. They are also heterogenous regarding their composition of epithelium, stroma, blood vessels and immune cells. As in our study, often no replicates can be run because of low sample amounts. Due to these factors an appreciable degree of variation is induced. An approach to overcome the latter problem may be the application of microdissection techniques in order to study gene expression in more homogenous endometrial specimens.

We further addressed the problem of intrinsic alterations of gene expression during explants culture in the absence of test compounds. Here significant alterations of target gene expression were observed after 6 h of culture in biopsies from all cycle stages. ER_{α} and PR mRNA levels were significantly down-regulated and LIF and COX-2 mRNA levels were up-regulated after 6 h of culture. We observed a high variation of basal gene expression in non-cultured samples from the same cycle phase. This reflects the dynamic change of gene expression within each cycle half (e.g. high expression of PR and ER in the late proliferative and early secretory phases, or of LIF during

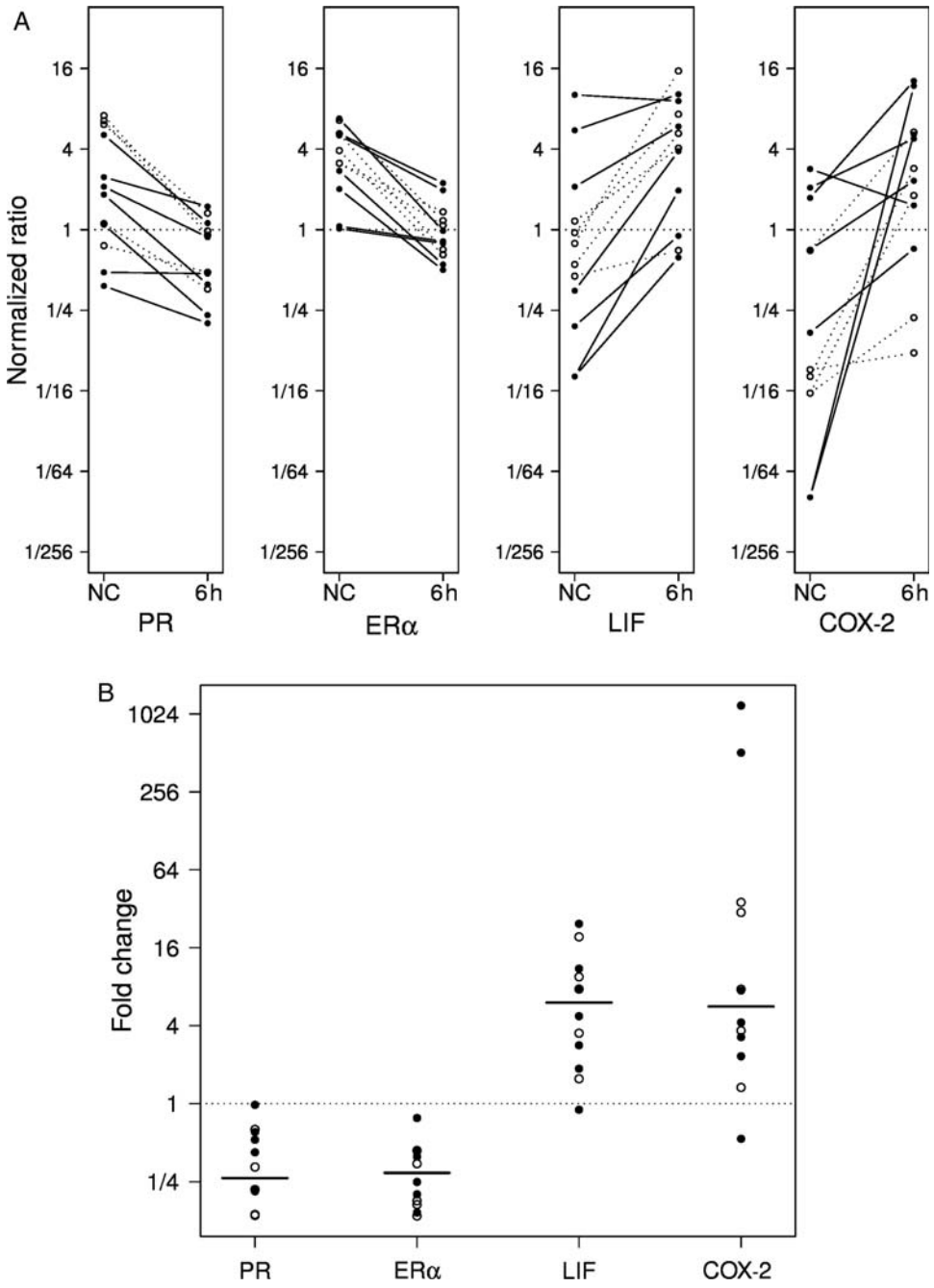

Figure 5 mRNA levels of endometrial target genes after 6 h explants culture compared with non-cultured biopsies. mRNA levels of PR, ER_α, LIF and COX-2 were determined by calibrator-normalized relative quantification in endometrial specimens ($n = 12$). PGK1 was used as reference gene. Explants were from different cycle phases and investigated non-cultured (NC) and after 6 h of culture in the presence of 17β-estradiol (10^{-9} M) and progesterone (10^{-7} M), mimicking the hormonal milieu in the secretory phase. Note that the *y*-axis is log_2-scaled, but tick labels are on the original scale. A normalized ratio of 1 is marked by horizontal dotted lines. (**A**) Line plots of normalized mRNA ratios for the different targets. In each line plot dots on the left side represent mRNA ratios in NC samples, dots on the right side represent mRNA ratios after 6 h of incubation. Results from the same patient are connected by dotted (proliferative phase) and solid (secretory phase) lines. (**B**) Alterations of mRNA levels for the different target genes during culture. Log_2-fold changes were calculated by subtraction of the respective log_2 values of normalized ratios in 6 h and NC samples. Fold change <1 indicates down-regulation, >1 up-regulation of the gene of interest during culture. For each target, the corresponding log_2 ratio is significantly different from 0 (signed rank test, unadjusted *P*-values <0.001). Open circles: proliferative phase, filled circles: secretory phase.

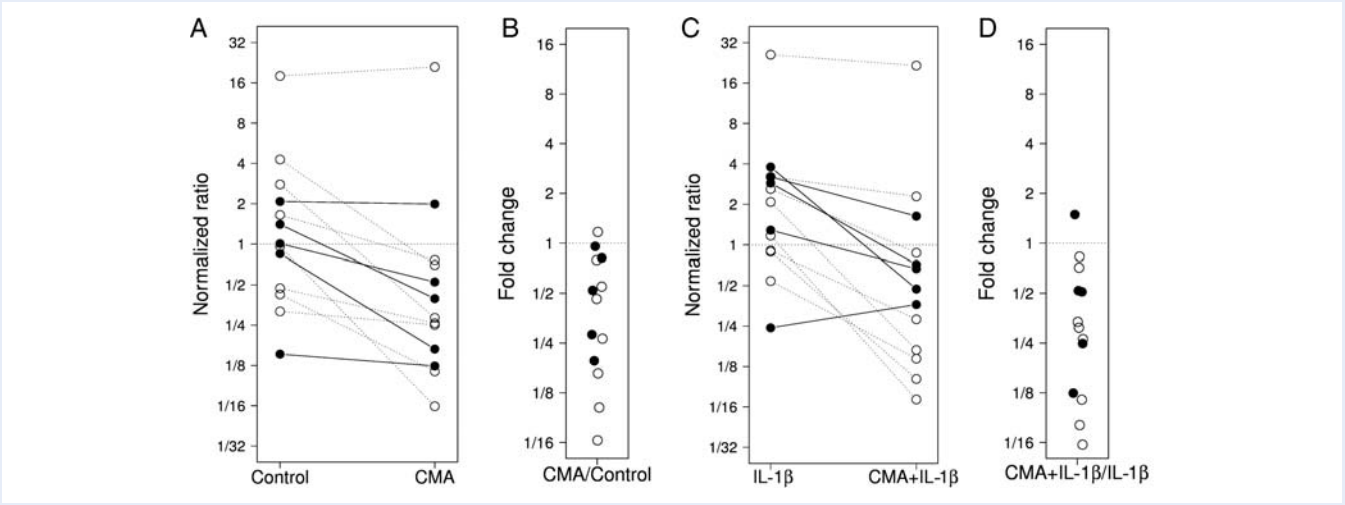

Figure 6 Effects of CMA on endometrial COX-2 mRNA levels. Effects of CMA (10^{-6} M) on COX-2 mRNA levels in unstimulated (**A** and **B**) and IL-1β (1 ng/ml) stimulated (**C** and **D**) endometrial explants from different cycle phases ($n = 13$). Controls contain vehicle alone (0.1% ethanol). Results are shown as normalized ratios (COX-2/G6PDH; A, C) and fold change (B, D). Note that the *y*-axes are $\log_2$-scaled, but tick labels are on the original scale. Results from the same patient are connected by dotted (proliferative phase) and solid (secretory phase) lines (A, C). A normalized ratio of 1 is marked by horizontal dotted lines. Lines connect results from the same patient. In the fold change diagrams (B, D) the relative COX-2 mRNA levels of individual samples are shown as circles (values <1 indicate down-regulation, >1 up-regulation). The $\log_2$-fold changes shown in B and D are significantly different from 0 ($P < 0.001$ in B; $P < 0.01$ in D). The upper outlier in (A) and (C) stems from a patient suffering from a *salpingitis isthmica nodosa* in the proximal tube, which may explain the high COX-2 mRNA levels in the endometrial specimen obtained by aspiration curettage. Open circles, proliferative phase; filled circles, secretory phase.

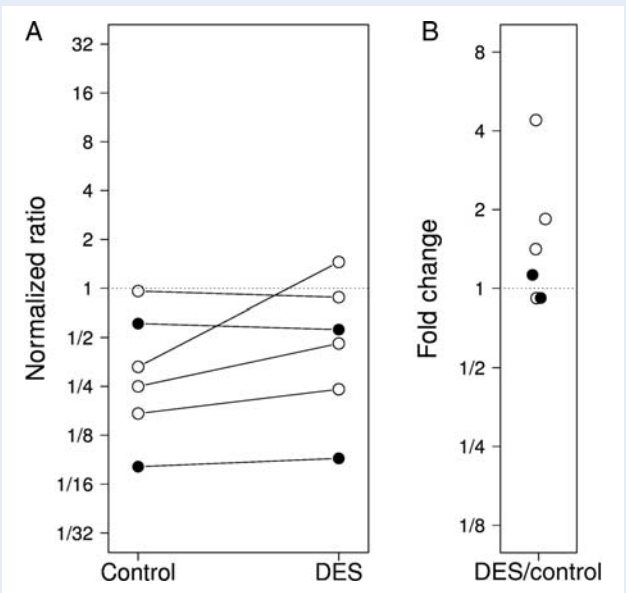

Figure 7 PR mRNA levels in endometrial explants under treatment with DES. Relative mRNA levels of PR in endometrial explants from different cycle phases after treatment with 10^{-6} M DES over 8 h in the absence of 17β-estradiol and progesterone are shown as normalized ratios (PR//G6PDH; **A**) and fold change (**B**). Solid lines connect results from the same patient. A normalized ratio of 1 is marked by horizontal dotted lines. Controls were incubated with vehicle only (0.1% ethanol). Open circles, proliferative phase; filled circles, secretory phase.

the window of implantation; Ingamells *et al.*, 1996). A further differentiation of the two-cycle phases was not realizable in our study since the low sample amounts did not permit parallel explants culture experiments and histological dating for all samples. A down-regulation of ER_α and PR after 8 h of culture has also been reported on the protein level (Stavreus-Evers *et al.*, 2003), confirming that expression of molecular targets is altered during culture in the absence of test substances. Similar culture-induced changes of gene expression have been found in other tissues (Gesta *et al.*, 2003). It is important to pay attention to the intrinsic alterations during explants culture since they indicate a discrepancy between *in vivo* and *ex vivo* situations. Therefore, as a minimal requirement matched control experiments without test compounds for each time point and biomarker, respectively, must be run.

In our experimental approach, RT-qPCR was used as a highly sensitive and rapid method to determine relative mRNA concentrations of pre-selected gene expression biomarkers in small-sized specimens of limited viability. Target and reference genes were investigated with pre-validated UPL assays, which cover approximately 99% of the human transcriptome with a set of 90 probes and making a broad spectrum of qPCR assays rapidly available. By use of the universally applicable UPL assays investigations can easily be extended to nearly all genes of interest.

The suitability of human endometrial explants cultures as *ex vivo* model for detecting functional effects of drugs and chemicals was addressed in two experiments. First, a significant down-regulation of COX-2 mRNA by CMA was demonstrated in unstimulated and IL-1β-stimulated samples from various menstrual cycle stages. This example demonstrates proof of principal that human endometrial

explants cultures can be used as model to characterize significant effects of exogenous test compounds on gene expression biomarkers. It is remarkable that after IL-1β challenge similar responses to CMA were observed in endometrial explants from both cycle halfs. However, the influence of menstrual cycle stage on effects of exogenous compounds should be explored for each specific experimental setting. The CMA effect observed in our study suggests an explanation for the beneficial effects of CMA-containing oral contraceptives in dysmenorrheic women (Anthuber *et al.*, 2010), probably due to its partial glucorticoidal activities (Rhen and Cidlowski, 2005; Druckmann, 2009; Schneider *et al.*, 2009). In a second experiment, endometrial PR mRNA levels were analyzed after treatment of explants from various cycle phases with the synthetic estrogen DES. In this experiment, no consistent up-regulation of PR mRNA was found in contrast to findings in the human endometrial Ishikawa cell line (Schaefer *et al.*, 2010) and to the well-known *in vivo* up-regulation of PR by estrogens (Jabbour *et al.*, 2006). High basal PR mRNA levels in non-cultured controls indicate that *in vivo* priming of the endometrium by endogenous estrogens prior to the *ex vivo* tests might attenuate the responsiveness towards exogenous estrogens. This result demonstrates the necessity to restrict tests with estrogenic compounds to explants from the proliferative phase.

From our point of view, the explants model, due to its intrinsic variability, is best applicable when strong effects are expected and the tissue is not exposed *in vivo* to the substance class investigated. For studying the effects of estrogens and progestins, patients from defined cycle stages should be selected. Dose–response relationships are hard to establish due to the small tissue amounts available.

We suggest that the primary applicability of endometrial explants is as a complementary research model. They may be useful for validation of screening tests with permanent cell lines (e.g. Ishikawa cells; Naciff *et al.*, 2010; Schaefer *et al.*, 2010). More advanced approaches like 3D models and 'artificial endometrium' are in an early stage of development (Fasciani *et al.*, 2003; Diedrich *et al.*, 2007; Lalitkumar *et al.*, 2007). However, the objective to study the highly complex and dynamic endometrial function with simple, standardizable *in vitro*-test systems remains a crucial challenge.

In conclusion, the advantages of using human explants are their close proximity to the *in vivo* situation due to the maintenance of tissue integrity and their derivation from different individuals. This is in contrast to tumor and immortalized cell lines, which differ from normal cells and tissues in many aspects. The drawbacks of explants cultures are their limited supply, cellular heterogeneity, contaminations, short viability, different menstrual cycle stage and interindividual variation. Therefore, functional studies should be conducted only over a limited time period and the impact of cycle stage should be evaluated. Our CMA experiment indicates that in specific experimental settings biopsies from different cycle stages display similar responses to exogenous compounds. Further, investigators should be aware of culture-induced alterations in gene expression, which indicate a discrepancy between *in vivo* and *ex vivo* situations. Anyhow, explants cultures are an important research tool and relevant for validation of findings obtained in cell culture experiments. RT-qPCR is a suitable and rapid method to investigate effects of exogenous substances on endometrial gene expression biomarkers during the limited *ex vivo* life span of this vulnerable tissue.

Authors' roles

W.R.S.: principal investigator, study design, data interpretation, manuscript preparation; L.F.: qPCR analysis, data collection and interpretation; K.R., A.K.J., J.E.S. and I.K. performed the explants culture experiments; P.S.: scanning electron microscopy; M.W.: statistical analysis. M.O.V.: histological evaluation; A.H.B.: study design, tissue sampling, data interpretation; W.R.D.: study design, data collection and interpretation; H.P.Z.: study design, data interpretation, supervision of the project.

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Conflicts of interest: H.P.Z.: Advisory board member for CMA, Grünenthal-International.

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