molecular
human
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ORIGINAL ARTICLE

Generation of immortalized human endometrial stromal cell lines with different endometriosis risk genotypes

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Endometriotic lesions are composed in part of endometrial-like stromal cells, however, there is a shortage of immortalized human endometrial stromal cultures available for research. As genetic factors play a role in endometriosis risk, it is important that genotype is also incorporated into analysis of pathological mechanisms. Human telomerase reverse transcriptase (hTERT) immortalization (using Lenti-hTERT-green fluorescent protein virus) took place following genotype selection; 13 patients homozygous for either the risk or non-risk 'other' allele for one or more important endometriosis risk single nucleotide polymorphism on chromosome 1p36.12 (rs3820282, rs56318008, rs55938609, rs12037376, rs7521902 or rs12061255). Short tandem repeat DNA profiling validated that donor tissue matched that of the immortalized cell lines and confirmed that cultures were genetically novel. Expression of morphological markers (vimentin and cytokeratin) and key genes of interest (telomerase, estrogen and progesterone receptors and LINC00339) were examined and functional assays for cell proliferation, steroid hormone and inflammatory responses were performed for 7/13 cultures. All endometrial stromal cell lines maintained their fibroblast-like morphology (vimentin-positive) and homozygous endometriosis-risk genotype following introduction of hTERT. Furthermore, the new stromal cultures demonstrated positive and diverse responses to hormones (proliferation and decidualisation changes) and inflammation (dose-dependent response), while maintaining hormone receptor expression. In conclusion, we successfully developed a range of human endometrial stromal cell lines that carry important endometriosis-risk alleles. The wider implications of this approach go beyond advancing endometriosis research; these cell lines will be valuable tools for multiple endometrial pathologies offering a level of genetic and phenotypic diversity not previously available.

Key words: endometrium / endometriosis / risk allele / stroma / cell culture / immortalization / hTERT

Introduction

Endometriosis is an estrogen-dependent gynaecological disorder that occurs in 6–10% of reproductive aged women (Giudice, 2010). Common symptoms of endometriosis include pelvic pain and sub-fertility. Endometriosis is characterized by lesions consisting of endometrial-like stroma and epithelium, found most commonly in the pelvis. The endometrial-like cells which form endometriotic lesions are thought to originate from eutopic endometrium, which is shed and expelled into the peritoneal cavity during retrograde menstruation (Sampson, 1925). Consequently, human endometrial stromal cells (hESC) are used routinely

in cell culture work for investigation of endometriosis. One limitation of cell culture with primary endometrial cells is obtaining adequate human tissue for research purposes. Furthermore, primary hESC vary in phenotype from batch to batch and are difficult to maintain in long-term culture as they gradually lose their endometrial stromal cell phenotype (Krikun et al., 2004; Barbier et al., 2005; Yuhki et al., 2011). To overcome these obstacles, primary cells can be immortalized, a process whereby cell division is prolonged through incorporation of human telomerase reverse transcriptase (hTERT) into the cells. At present there is only one reliable

immortalized hESC line transfected with hTERT that is commercially available ([Krikun et al., 2004](#)).

While the cellular mechanisms responsible for the development of endometriotic lesions are largely unknown, there is an established genetic component to the disease. Genome-wide association studies (GWAS) have identified genomic regions associated with increased endometriosis risk ([Fung et al., 2015b](#); [Sapkota et al., 2017](#)). Expression quantitative trait loci (eQTL) are essential to understanding the functional implications of GWAS. An eQTL is a polymorphism that is associated with transcript abundance and therefore, eQTLs may provide clues as to how polymorphisms affect gene expression in a trait or disease ([Gibson et al., 2015](#)). There are several published eQTLs associated with endometriosis ([Fung et al., 2015a](#), [2017](#), [2018](#); [Holdsworth-Carson et al., 2016](#)), and most recently we demonstrated a strong tissue-specific eQTL at 1p36.12 for decreased expression of a long intergenic non-protein coding RNA 339 (*LINC00339*) in the endometrium ($P = 2.4 \times 10^{-8}$) ([Powell et al., 2016](#)). Studying the genetic landscape of women with endometriosis is essential if we are to improve our understanding of the altered cellular mechanisms involved in disease development.

There is a shortage of suitable human cell culture models for endometriosis research; consequently, an understanding of the cellular mechanisms that drive this common gynaecological disease is lacking. The aim of this work is to generate and characterize a set of immortalized hESC lines derived from women with defined endometriosis-associated single nucleotide polymorphisms (SNPs) on chromosome 1p36.12. Moving forward, these immortalized cell lines will be an invaluable *in vitro* tool for studies of endometriosis providing researchers with variations in genetic background not previously available.

Materials and Methods

Tissue collection and consent

Endometrial tissue samples were collected by curettage from women recruited through the Royal Women's Hospital (Melbourne). Women undergoing laparoscopy provided informed written consent before surgery. Ethical approval for the study was obtained from the Human Research Ethics Committee (Projects 10-43 and 11-24) of the Royal Women's Hospital. In addition to providing a blood sample and tissues; clinical, surgical and pathology information was collected for each participant. A portion of each endometrial biopsy was evaluated by an experienced gynaecological pathologist and classified either according to menstrual cycle stage, or as inactive, influenced by exogenous progestins, or as unknown (unable to confidently classify based on available tissue). It was our priority to generate stromal cell lines from women with important endometriosis-associated SNPs (specific to chromosome 1p36.12), of which the homozygous risk allele (RA) occurred at a low frequency; therefore, we were not able to control for contraception use or menstrual cycle stage. Endometriosis or non-endometriosis status was allocated following detailed surgical investigation based on the revised American Fertility Society (rAFS) or the American Society for Reproductive Medicine definitions ([Medicine, 1997](#)) as determined by the surgeon. Samples were de-identified and designated an internal 4-digit identifying number that was used to distinguish the different cell lines. Endometrial curettes were collected in theatre into 20 ml of chilled Dulbecco's Modified Eagle Medium/Nutrient Mixture F12, HEPES (DMEM/F12, HEPES) (with 5% (v/v) new born calf serum and 1x antibiotic–antimycotic) (Thermo Fisher Scientific, Scoresby, Australia). Samples were stored at 4°C and processed immediately or the following morning (within 24 h). A total of 39 primary hESC cultures were established and stored over a 12-month period.

SNP genotyping

DNA from whole blood, endometrial tissue and cell cultures were extracted using QIAGEN DNeasy Blood & Tissue extraction kits (Hilden, Germany). DNA samples were genotyped on HumanCoreExome chips (Illumina, Inc., San Diego, CA, United States). Standard quality control procedures were applied as outlined previously ([Luong et al., 2013](#)). Patient genotypes were examined to select appropriate primary stromal cell cultures for hTERT immortalization. Of the 39 patients, 13 were selected because they were homozygous for either the RA or the non-risk 'other' allele (OA) for at least one specific endometriosis-associated SNP on chromosome 1 (1p36.12). These SNPs included rs12037376, rs3820282, rs56318008, rs55938609 and rs7521902 ([Powell et al., 2016](#)). Although not an endometriosis risk SNP, rs12061255 was also included for selection as it showed a significant eQTL for *LINC00339* expression. SNP rs12037376 at the 1p36.12 locus has the largest effect size overall on endometriosis risk (P-value 8.87×10^{-17}) ([Sapkota et al., 2017](#)). All of the SNPs on chromosome 1, except for rs12061255, are in high or moderate linkage disequilibrium (LD) (in Europeans, LD between the four SNPs rs56318008, rs55938609, rs12037376 and rs3820282 are high with $R^2 > 0.9$, and rs7521902 has an R^2 of ~0.5 with these four SNPs) ([Powell et al., 2016](#)).

The genotypes of hTERT immortalized hESC cultures for each patient were also compared with the genotypes of the matched whole blood and endometrial tissue to demonstrate genotype stability.

Primary culture of hESC

Human ESC were isolated as described previously ([Dimitriadis et al., 2002](#)), with some modifications. Briefly, endometrial tissue was rinsed with phosphate buffered saline (PBS), manually minced and enzymatically digested with 0.375 mg/ml Collagenase III (Sigma-Aldrich, Castle Hill, Australia), 25 µg/ml DNase I (Roche, Hawthorn, Australia) and 10 mg/ml glucose (Sigma-Aldrich) in PBS at 37°C in a shaking incubator. When a single cell suspension was obtained it was filtered sequentially through 45 and 10 µm filters (Allied Filter Fabrics, Hornsby, Australia) to remove epithelial cells. (*Note*: We were unable to produce sufficient yield (coupled with poor survival) and purity of epithelial cells to pursue immortalization of these cultures.) Red blood cells were lysed with ACK (Ammonium-Chloride-Potassium) lysing buffer as per manufacturer's instructions (Thermo Fisher Scientific). The endometrial stromal cell suspension was pelleted and resuspended in culture media (DMEM/F12 / 10% (v/v) foetal calf serum (FCS)/1x insulin-transferrin-selenium-ethanolamine (ITS-X)/1x antibiotic–antimycotic (Thermo Fisher Scientific)). Primary hESC, passage 0 (P0), were cultured in 25 cm^2 flasks and chamber slides at 10 000 cells/cm^2 in a humidified CO_2 incubator at 37°C. Flasks of primary P0 hESC were grown to confluence, trypsinised, frozen and stored in liquid nitrogen until SNP genotypes were determined, and appropriate cultures could be selected for immortalization.

hTERT immortalization of hESC

Selected endometrial stromal cells were thawed and plated at passage 1 (P1) in three wells of a 48-well plate (0.25×10^5 cells per well) in culture media for 24 h until 50% confluent. Cells were immortalized using a Lenti-hTERT-green fluorescent protein (GFP) virus (Applied Biological Materials Inc., Richmond, Canada) with ViralPlus Transduction Enhancer (1:50 dilution) (Applied Biological Materials Inc.) and polybrene (8 µg/ml) (Merck Millipore, Bayswater, Australia). The amount of Lenti-hTERT-GFP virus for successful transfection was determined empirically; a multiplicity of

infection (MOI) of 200 was determined to be most efficient and employed in these studies. Controls were included in the second and third wells; a blank GFP virus (Lenti-CMV-GFP-2A-Puro, Applied Biological Materials Inc.) and a no-virus control. Twenty-four hours following transfection, the media was replaced. Between 2 and 7 days post-transfection, cells were monitored for lentivirus vector uptake (GFP expression) using an inverted fluorescent microscope (Olympus, Notting Hill, Australia). hTERT transfected hESC cultures were grown to near confluence before media containing puromycin (0.75 μg/ml) (Thermo Fisher Scientific) was added. Drug selection occurred over 5 days, which was sufficient time to kill non-transfected cells including the no-infection control. Puromycin resistant hTERT immortalized hESC were grown to confluence in puromycin-free DMEM/F12/20% FCS/1x ITS-X/1x antibiotic–antimycotic until the next passage. The hTERT hESC were then maintained in DMEM/F12/10% FCS/1x ITS-X/1x antibiotic–antimycotic. A specialized fibroblast media, Medium 106 (M106) plus 1x Low Serum Growth Supplement (LSGS) and 1x antibiotics–antimycotic (Thermo Fisher Scientific), was also employed on some of the new hTERT lines.

Culture of telomerase-transformed HESC line

Transformed HESCs (T HESC) were a gift to PAWR from Dr Graciela Krikun (Yale University) (Krikun et al., 2004) and were maintained in DMEM/F12/10% FCS/1x ITS-X/1x antibiotic–antimycotic. Culture media was refreshed every 2–3 days and cells were passaged every 4–6 days with trypLE express (Thermo Fisher Scientific). The T HESC line was used to compare with the newly established hTERT hESC cultures against steroid receptor expression and in vitro assays.

Immunocytochemistry

Primary P0 endometrial stromal cultures grown in chamber slides were fixed with 4% (w/v) paraformaldehyde and stained for vimentin (mouse monoclonal antibody [clone V9]; Thermo Fisher Scientific) and cytokeratin (mouse monoclonal antibody; Agilent, Mulgrave, Australia) to confirm purity of isolations; stromal cells were positive for vimentin and negative for cytokeratin (Dimitrov et al., 2008). Following several passages the GFP control and hTERT immortalized endometrial stromal cell cultures were grown in chamber slides and examined by immunocytochemistry (ICC) for vimentin, cytokeratin and steroid hormone receptors estrogen receptor alpha (ESR1) (mouse monoclonal antibody [clone 6F11]; Abcam, Melbourne, Australia), estrogen receptor beta (ESR2) (rabbit monoclonal antibody [clone EPR3778]; Abcam) and progesterone receptor (PR) (rabbit monoclonal antibody [clone D8Q2J]; Cell Signaling Technology, Arundel, Australia). The appropriate isotype controls (Agilent) were included in each ICC experiment.

Fixed cells were washed (PBS) and permeabilized with 0.1% triton X-100 in PBS for 30 min at room temperature (RT). Peroxidases were blocked with 0.3% H_2O_2 for 10 min at RT then non-specific protein were blocked using Protein Block, serum-free (Agilent) for 10 min, RT. Antibodies and isotype controls in 1% (w/v) BSA (Sigma Aldrich) were incubated overnight at 4°C. Antibody concentrations were as follows; vimentin 0.14 μg/ml, cytokeratin 0.18 μg/ml, ESR1 0.63 μg/ml, ESR2 2.2 μg/ml and PR 0.63 μg/ml. REAL EnVision Detection System with Peroxidase/DAB+ chromogen (Agilent) was used for visualization. ICCs were counterstained with Mayer's haematoxylin for 1 min and mounted with aqueous Ultramount Permanent Mounting Medium (Agilent).

The percentage of vimentin-, cytokeratin-, ESR1-, ESR2- and PR-positive cells was calculated by counting the number of brown-stained cells or brown-stained nuclei relative to the total number of haematoxylin-stained cells in five fields of view (centre, top left, top right, bottom left and bottom right).

Short tandem repeat DNA profiling

Short tandem repeat (STR) profiling was performed on the 13 new hTERT endometrial stromal cell lines, matching donor endometrial tissue and the T HESC line. STR was used to confirm integrity of the cells against the matched donor tissue and novelty of each line from other existing cell lines. Profiling was also used to ascertain a unique STR DNA profile for each new cell line for any future comparisons/authentication.

Frozen endometrial tissue (10–20 mg) and cell pellets (1 million cells) were provided to the Garvan Institute of Medical Research (Darlinghurst, NSW, Australia) for DNA extraction and subsequent STR profiling using the PowerPlex 18D System (which reports allele calls for 18 different STR loci; D5S818, D13S317, D7S820, D16S539, vWA, TH01, Amel, TPOX, CSF1PO, D8S1179, D21S11, D3S1358, D2S1338, D19S433, D18S51, FGA, Penta E and Penta D). Profiles were analysed against three databases (biorepository agencies ATCC and DSMZ, and an internal Garvan database) and samples were considered to match if their profiles were more than 80% identical. STR DNA profiling authenticated the T HESC line, demonstrating a STR profile identical to T HESCs ATCC-CRL-4003.

Proliferation assay

The T HESC line and new hTERT cultures (1399, 1405, 1429, 1433, 1455 and 1458) were examined for cell proliferation using the RealTime Glo MT Cell Viability Assay (Promega, Alexandria, New South Wales, Australia), following the manufacturer's instructions. Prior to seeding, all cell lines were cultivated in DMEM/F12 (as described above) except for 1399 and 1405, which were grown in M106. Since 1455 cells grew well in both types of media, 1455 cells originating from both DMEM/F12 and M106 medium were included in proliferation assays. Single cell suspensions were seeded into 96-well plates (1000–4000 cells/well) in phenol red-free DMEM/F12/5% (charcoal stripped) csFCS/1x ITS-X/1x antibiotics–antimycotic and incubated overnight in a humidified CO_2 incubator at 37°C. The following day, estradiol 17-β (E, estrogen), medroxyprogesterone acetate (MPA) and estrogen + MPA combined were added to the wells at a final concentration of 10^{-5} M (Sigma-Aldrich). A vehicle control was also included (0.01% DMSO) per cell line. Luminescence readings were taken using a FLUOstar Omega microplate reader (BMG Labtech, Mornington, Victoria, Australia). Fold change in luminescence was calculated every 24 h for 6 days. The growth rate was calculated as LN (fold change N_1/fold change N_2)/total N, where N = day. Cell doubling time was calculated on Day 3 using the following formula, LN (2)/growth rate. Assays were repeated twice in triplicate. Hormone-treated proliferation data is displayed as a fold-change relative to vehicle for Days 1 and 3, normalized to Day 0.

Decidualisation assay

Aliquots from a subset of new hTERT endometrial stromal cell lines (1399, 1405, 1429, 1433, 1455 and 1458) were seeded into 12-well plates and decidualized over 72 h in the presence of estrogen (10^{-8} M), MPA (10^{-7} M) and cAMP (0.01 M) (all from Sigma-Aldrich) in DMEM/F12/2% csFCS/1x ITS-X/1x antibiotic–antimycotic or M106/serum-free/1x antibiotics–antimycotic (Yuhki et al., 2011; Kommagani et al., 2016). Vehicle controls were also included for each experiment. Cell morphology was observed before and after decidualisation. Cells were collected and stored at −80°C for RNA extraction, specifically to examine expression levels of decidualisation markers insulin-like growth factor-binding protein 1

(*IGFBP1*) and prolactin (*PRL*). Decidualisation was performed in triplicate per cell line.

Inflammatory response assay

Aliquots from a subset of new hTERT endometrial stromal cell lines (1399, 1405, 1429, 1433, 1455 and 1458) and the T HESC line were seeded in 12-well plates (40 000 cells/well) and grown to ~80% confluence before they were serum starved overnight (DMEM/F12/1% csFCS/1x ITS-X/1x antibiotics–antimycotic or M106/serum-free/1x antibiotics–antimycotic). The following day, cells were treated with lipopolysaccharide (LPS) (10, 100 and 10 000 ng/ml) (Sigma-Aldrich) or vehicle (PBS) for 24 h (Rashidi *et al.*, 2015), before cells were collected and stored at −80°C for RNA extraction. Assays were performed in triplicate per cell line.

RNA extraction, cDNA synthesis and real-time PCR

RNA was extracted using Direct-zol RNA MicroPrep kits (Zymo Research, California, USA) or ReliaPrep RNA Cell Miniprep kits (Promega) following the manufacturer's instructions. RNA concentrations were quantified using a NanoDrop (Thermo Fisher Scientific). RNA was converted to cDNA using Affinity Script QPCR cDNA Synthesis kits (Agilent).

Real-time PCR experiments were performed using a 7500 Real-Time PCR instrument and TaqMan assays (Thermo Fisher Scientific). The following FAM TaqMan assays were used: *TERT* (#Hs00972560_m1), *LINC00339* (#Hs04402696_m1), *ESR1* (#Hs00174860_m1), *ESR2* (#Hs0023095 7_m1), *PR* (#Hs01556702_m1), *IGFBP1* (#Hs00236877_m1), *PRL* (#Hs001 68730_m1), *CXCL8* (#Hs00174103_m1), *IL1B* (#Hs01555410_m1), *NFKB1* (#Hs00765730_m1), *CXCL5* (#Hs01099660_g1), *B2M* (#Hs0018784 2_m1) and *RPL13A* (#Hs01926559_g1). Two microliters of diluted cDNA (dilution 1:20) was used per 10 μl reaction using Brilliant III Ultra-Fast QRT-PCR Master Mix (Agilent). Quantification of gene expression were normalized to the average C_t values of human beta-2-microglobulin (*B2M*) and ribosomal protein L13a (*RPL13A*) housekeeping genes using the comparative threshold cycle method. *B2M* and *RPL13A* were selected as they were stably expressed, non-hormone responsive and demonstrated negligible variability (data not shown).

Statistics

Statistical analysis was performed using GraphPad Prism (version 7, GraphPad software, CA, USA). Expression data (ICC) and LPS-response experiments were analysed by ANOVA with Tukey's post hoc test. Decidualisation and hormone-treated proliferation data were analysed using *t* tests. A *P*-value <0.05 was considered statistically significant for all analyses.

Results

Patient selection and SNP genotyping

We selected cells from 13 patients who were homozygous (RA or OA) for at least one of six endometriosis-associated SNPs on chromosome 1p36.12 (rs3820282, rs56318008, rs55938609, rs12037376, rs7521902 or rs12061255) to undergo hTERT immortalization. Due to the lower allele frequency of RA variants in the population, cell lines were more likely to be homozygous for an OA SNP relative to the RA genotype. A list of patient characteristics is provided in Table I. The 10 out of 13 women had surgically confirmed endometriosis, 12 out of 13 reported severe menstrual pain (or dysmenorrhoea) and 11 out of 13 reported chronic pelvic pain. Women had various other common

Table I Patient characteristics.

Characteristic		
	Median (range)	
Age (years)	32 (23–41)	
Age at menarche (years)	13 (11–17)	
Gravidity	0 (0–3)	
Parity	0 (0–2)	
BMI (kg/m^2)	25 (19.3–39.6)	
	Percentage (n*)	
Current smoker	17% (2)	
Previous smoker	46% (6)	
Cycle stage	Mid proliferative	31% (4)
	Early Secretory	15% (2)
	Late Secretory	8% (1)
	Progestin	15% (2)
	Inactive	15% (2)
	Unknown	15% (2)
Hormone therapy	None	54% (7)
	Mirena	8% (1)
	OCP	38% (5)
Endometriosis**	77% (10)	
Stage of endometriosis (*n* = 10 with endometriosis)	Stage I	40% (4)
	Stage II	30% (3)
	Stage III	0% (0)
	Stage IV	30% (3)
rAFS score (median, range)	10 (1–135)	
Severe menstrual pain	92% (12)	
Severe pelvic pain	85% (11)	
Dysparunia	77% (10)	
Uterine fibroids^	31% (4)	
Adenomyosis^	54% (7)	
Uterine polyp^	8% (1)	
Previous cervical cancer/precancer^	15% (2)	
PCOS^	23% (3)	

*n = 13 unless otherwise stated. **Visually confirmed by the surgeon, but not by pathology. ^Self-reported and/or clinically reported. rAFS = revised American Fertility Society; BMI = body mass index; PCOS = polycystic ovarian syndrome; OCP = oral contraceptive pill.

gynaecological co-morbidities (e.g. 31% reported uterine fibroids and 54% reported adenomyosis) and 6 out of 13 women reported that they were concurrently taking hormone medications.

Use of SNP genotyping confirmed that the genotypes of the 13 immortalized hESC lines matched those of the original donors (whole blood and endometrial tissue) (data not shown), demonstrating that the immortalization process did not alter the original SNP makeup of the cells. In addition to SNPs at the 1p36.12 risk loci, thirteen other genomic regions harbour significant risk loci for endometriosis (Sapkota *et al.*, 2017). Therefore, the hTERT hESC lines were examined for key endometriosis risk SNPs present on chromosomes 2, 4, 6, 7, 9, 11 and 12. Table II displays the RA and OA status of all 14 key

Table II The presence of homozygous risk alleles (RA) or other risk alleles (OA) for 14 key SNPs with genome wide significance for endometriosis.

Associated gene	Cytoband	SNP	RA Percentage % (n)	OA	Het.
WNT4	1p36.12	rs12037376	15% (2)	77% (10)	8% (1)
GREB1	2p25.1	rs11674184	31% (4)	15% (2)	54% (7)
ETAA1	2p14	rs6546324	0% (0)	69% (9)	31% (4)
IL1A	2q13	rs10167914	8% (1)	62% (8)	31% (4)
FN1	2q35	rs1250241	8% (1)	31% (4)	62% (8)
KDR	4q12	rs1903068	31% (4)	31% (4)	38% (5)
ID4	6p22.3	rs760794	31% (4)	38% (5)	31% (4)
CCDC170	6q25.1	rs1971256	8% (1)	46% (6)	46% (6)
SYNE1	6q25.1	rs71575922	0% (0)	62% (8)	38% (5)
–	7p12.3	rs74491657	69% (9)	0% (0)	31% (4)
CDKN2B-AS1	9p21.3	rs1537377	46% (6)	15% (2)	38% (5)
FSHB	11p14.1	rs74485684	77% (10)	0% (0)	23% (3)
VEZT	12q22	rs4762326	38% (5)	8% (1)	54% (7)
–	7p15.2	rs12700667	69% (9)	0% (0)	31% (4)

RA, risk allele; OA, other risk allele; Het., heterozygous; SNP, single nucleotide polymorphism.

Table III Short tandem repeat (STR) DNA profile results for each hTERT hESC line relative to its matching donor endometrial tissue.

	1399	1399	1400	1400	1405	1405	1406	1406	1415	1415	1418	1418	1429	1429
	Cells	Tissue	Cells	Tissue	Cells	Tissue	Cells	Tissue	Cells	Tissue	Cells	Tissue	Cells	Tissue
Match *	100%		94%		100%		100%		97%		97%		100%	
	1433	1433	1440	1440	1441	1441	1448	1448	1455	1455	1458	1458		
	Cells	Tissue	Cells	Tissue	Cells	Tissue	Cells	Tissue	Cells	Tissue	Cells	Tissue		
	97%		100%		100%		100%		100%		100%			

*The degree of identity matching between the cell line and the original donor tissue. Cell lines and donor tissue are considered to be a match if their profiles are more than 80% identical.

SNPs with genome wide significance for endometriosis in our 13 cell lines (note that only SNP rs12037376 is listed for 1p36.12 as per Sapkota et al., 2017).

STR DNA profiling

All 13 newly established immortalized hESC lines were found to have unique STR profiles (data not shown). The STR profile of the donor endometrial tissue was analysed against its matched hTERT hESC line, and each cell line was confirmed to match the tissue of origin (matches ranging between 94 and 100%) (Table III).

hTERT immortalized endometrial stromal cell cultures: morphology and expression of genes of interest

Figure 1 depicts GFP-positive hESC following the successful introduction of lenti-hTERT-GFP and 5 days of puromycin drug-selection. The immortalization process did not impact on stromal cell marker expression, with hTERT immortalized stromal cells maintaining their vimentin-positive and cytokeratin-negative status (Fig. 2a–d). The endometrial stromal cell cultures maintained a fibroblast-like appearance before and after immortalization (Fig. 2e–i). Cells were spindle (bipolar) or stellate (multipolar) shaped and grew in parallel arrangements when near confluence. In contrast to the T HESC line (Fig. 2j), the hTERT hESC were larger in size (Fig. 2e–i).

The uptake of the lenti-hTERT was further validated by RT-PCR. Telomerase (TERT) mRNA was detected in all the newly established cell lines as well as the T HESC line (Fig. 3). We then examined the cell lines (and primary cultures) for the expression of steroid hormone receptors. The range in expression levels was variable, however, ESR1, ESR2 and PR mRNA were detected in all the hTERT immortalized endometrial stromal cell lines and median expression levels were not significantly different compared to the primary (P0) cell cultures (Fig. 3). Nuclear protein expression of the hormone receptors (ESR1,
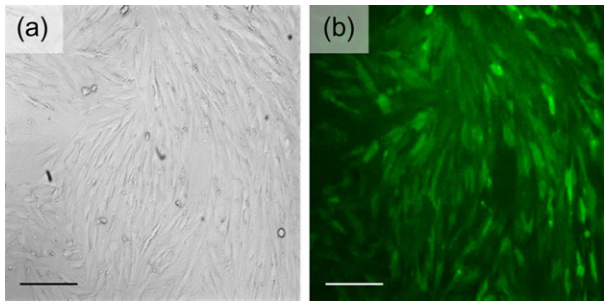
Figure 1 Representative image of human endometrial stromal cells (passage [P] 2) following immortalization with the lenti-human telomerase reverse transcriptase (hTERT)-green fluorescent protein (GFP) virus (multiplicity of infection [MOI] of 200) and subsequent puromycin treatment. The same field of view captured in (**a**) brightfield and (**b**) fluorescence demonstrating GFP positivity. Scale bar = 100 μm.

ESR2 and PR) was also confirmed by ICC (Fig. 4a–c). The median level of protein expression in immortalized cells was not different to the median levels found in primary (P0) cells (Fig. 4d–f). The strongest endometrial eQTL at 1p36.12 is for *LINC00339* (Powell *et al.*, 2016) and we report that all hESC lines express *LINC00339* mRNA at varying levels (Fig. 5).

Proliferation of hTERT immortalized endometrial stromal cell lines

The new hTERT hESC lines displayed variable growth rates. To improve growth ability, the cells were grown in specialized M106 fibroblast media. Some of the cell cultures grew better in M106 media compared to the original DMEM/F12 media (1399, 1405, 1415, 1418, 1441 and 1448), while others did not (1406, 1429, 1433, 1440 and 1458). 1400 and 1455 grew equally in both types of culture media.

Proliferation assays were performed on a subset of cell lines that were observed to have encouraging growth rates (1399, 1405, 1429, 1433, 1455 [both media types] and 1458) relative to the T HESC line.
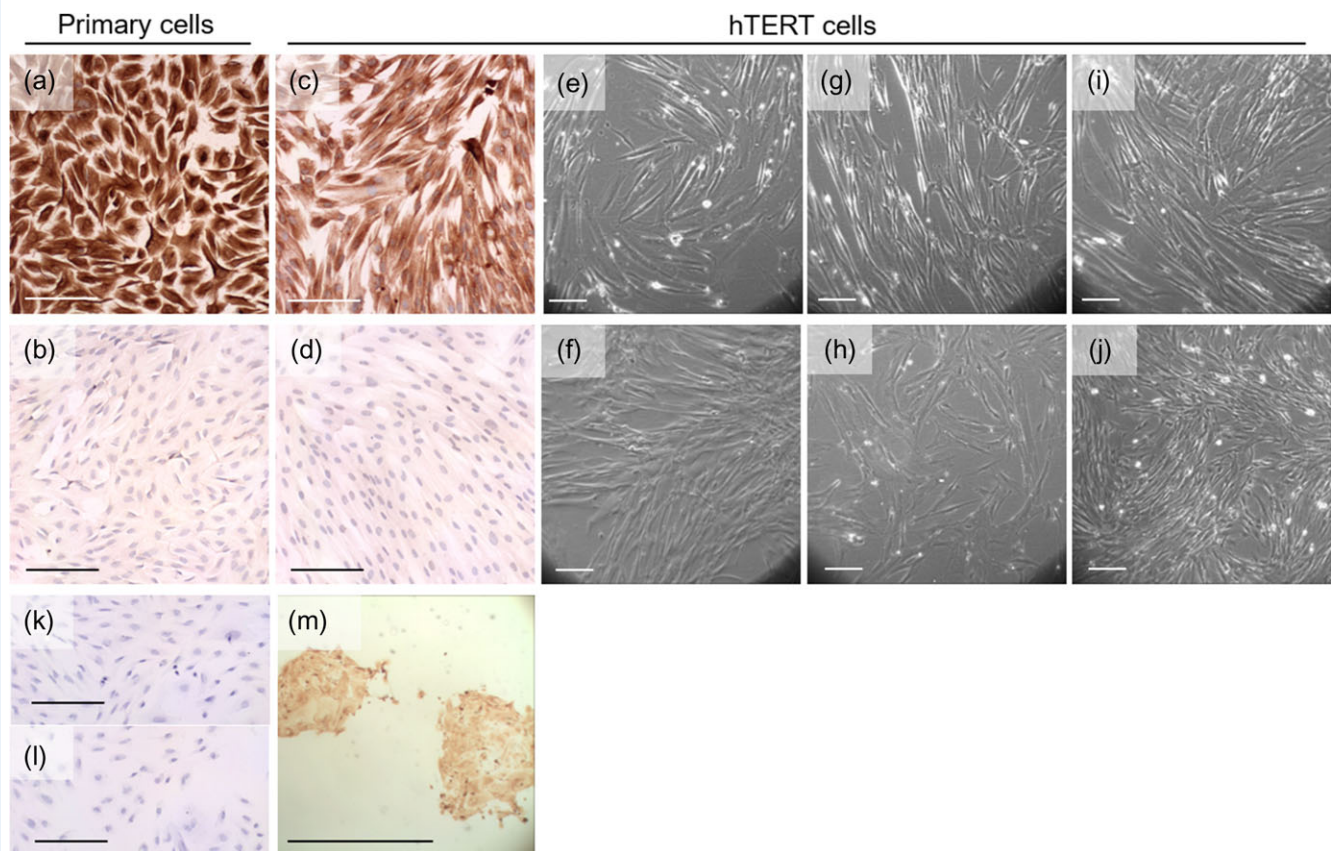

Figure 2 Representative images of human endometrial stromal cells which were vimentin-positive (a and c) and cytokeratin-negative (b and d). Primary stromal cells (P0) are shown in (**a**) and (**b**) and hTERT immortalized stromal cells (P6) are shown in (**c**) and (**d**). Brightfield images demonstrate the fibroblast-like morphology of hTERT immortalized human endometrial stromal cell cultures (**e–i**) relative to the telomerase-transformed human endometrial stromal cell (T HESC) line (**j**). No antibody control (**k**), IgG1 isotype control (**l**) and cytokeratin positive control showing brown staining of primary (P0) epithelial cells (**m**). Scale bar = 100 μm.
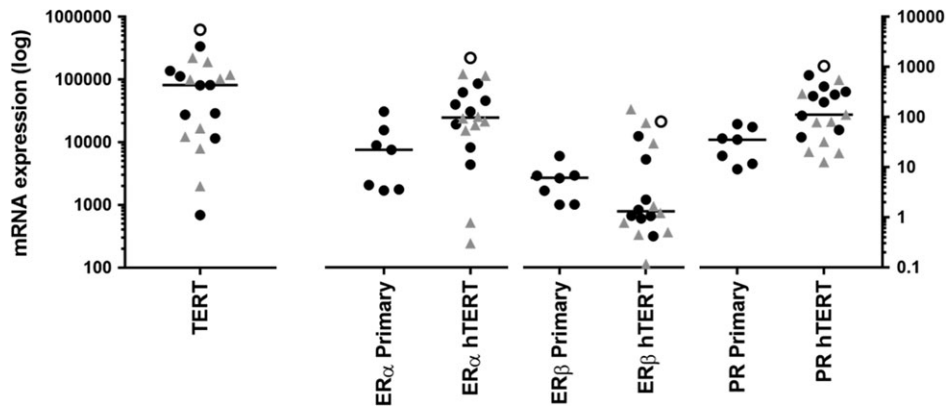
Figure 3 **mRNA expression of *TERT*, estrogen receptor alpha (*ESR1*), estrogen receptor beta (*ESR2*) and progesterone receptor (*PR*) in primary (P0) and hTERT immortalized ESC (between P3 and P7) (relative to housekeeping genes).** Median expression of each gene is indicated by the black bar. Left-hand *y*-axis is for *TERT*, right-hand *y*-axis is for *ESR1*, *ESR2* and *PR*. Closed black circles; cells grown in Dulbecco's Modified Eagle's medium (DMEM)/12 media, open circles; T HESC and grey triangles; cells grown in M106 media.
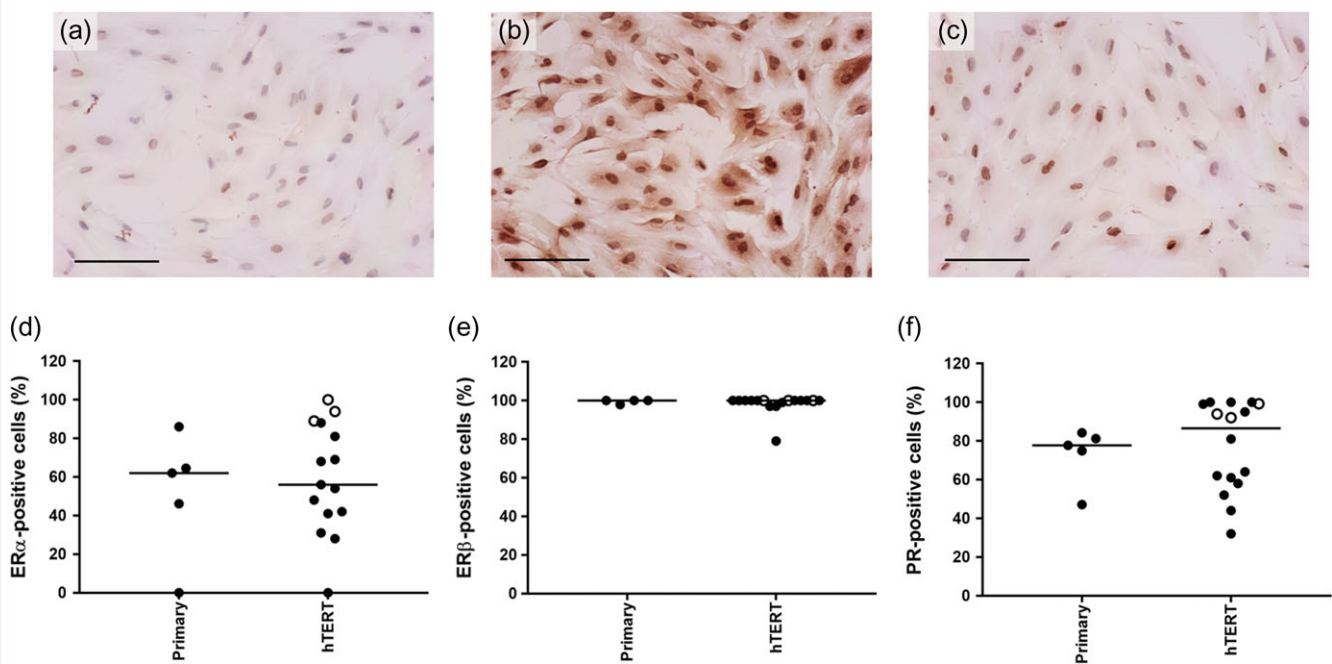
Figure 4 **Protein expression of hormone receptors in primary (P0) and human hTERT immortalized endometrial stromal cells (ESC).** Primary cultures distinct from the hTERT hESC lines (*n* = 4 or 5), were employed for immunocytohistochemistry (ICC) analysis. Representative images of hTERT cells stained for estrogen receptor alpha (ESR1) (**a**), estrogen receptor beta (ESR2) (**b**) and progesterone receptor (PR) (**c**) where brown equals positive staining. Scale bar = 100 μm. The percentage of positive cells are graphed for ESR1 (**d**), ESR2 (**e**) and PR (**f**) with median expression indicated by the black bar.

The fastest growing cells were 1455 (growing in DMEM/F12 and M106) with doubling times of 1.13 and 1.15 days, respectively, then followed by 1429 with a doubling time of 1.31 days (Fig. 6). The slowest in the group was 1433, with a doubling time of 2.47 days. The T HESC cell line had a doubling time of 1.26 days (Fig. 6a). Proliferation increased in a linear fashion over 3 days for all cell lines (Fig. 6b). Cells continued to increase linearly over time, except for 1433 and 1458 which plateaued by Day 6 (Fig. 6b). We report that several of these cell lines have been grown beyond passage 20 (including 1455, 1458 and 1433). Some of the new cell lines (and the T HESC line) demonstrated a significant increase fold-change in cell proliferation in the presence of estrogen relative to vehicle (Fig. 6c, d and h). In contrast,
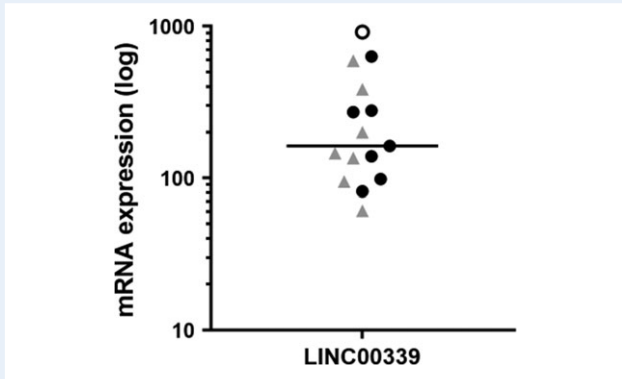
Figure 5 mRNA expression of long intergenic non-protein coding RNA 339 (*LINC00339*) in hTERT immortalized endometrial stromal cells (between P3 and P7) (relative to housekeeping genes). Closed black circles; cells grown in DMEM/12 media, open circle; T HESC and grey triangles; cells grown in M106 media.

MPA treatment (alone or combined with estrogen) reduced cell proliferation compared to vehicle-treatment in some cultures (Fig. 6d, e, g and h).

Decidualisation capacity of hTERT immortalized endometrial stromal cell lines

A decidual cell appearance developed in 1399 and 1405 cell lines, but not in the remaining immortalized cell lines following the 72 hr decidualisation protocol (Fig. 7a and b). Following decidualisation treatment however, all the cell lines demonstrated a significant increase in decidual cell-associated markers *IGFBP1* and/or *PRL* mRNA expression relative to vehicle (Fig. 7c and d). Two cell lines (1399 and 1433) did not demonstrate an increase in *IGFBP1* mRNA in response to decidualisation, however, both cell lines showed a significant increase in *PRL* expression following decidualization.
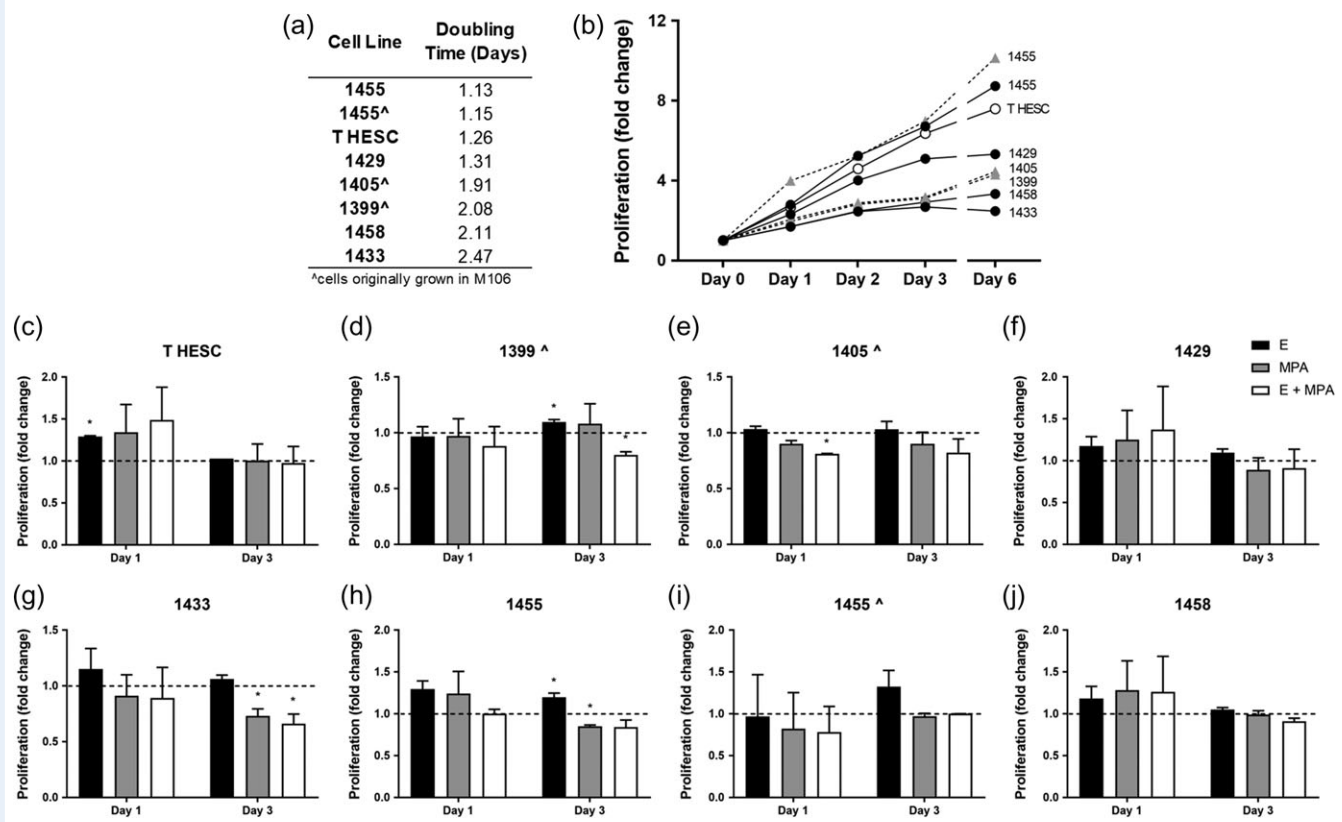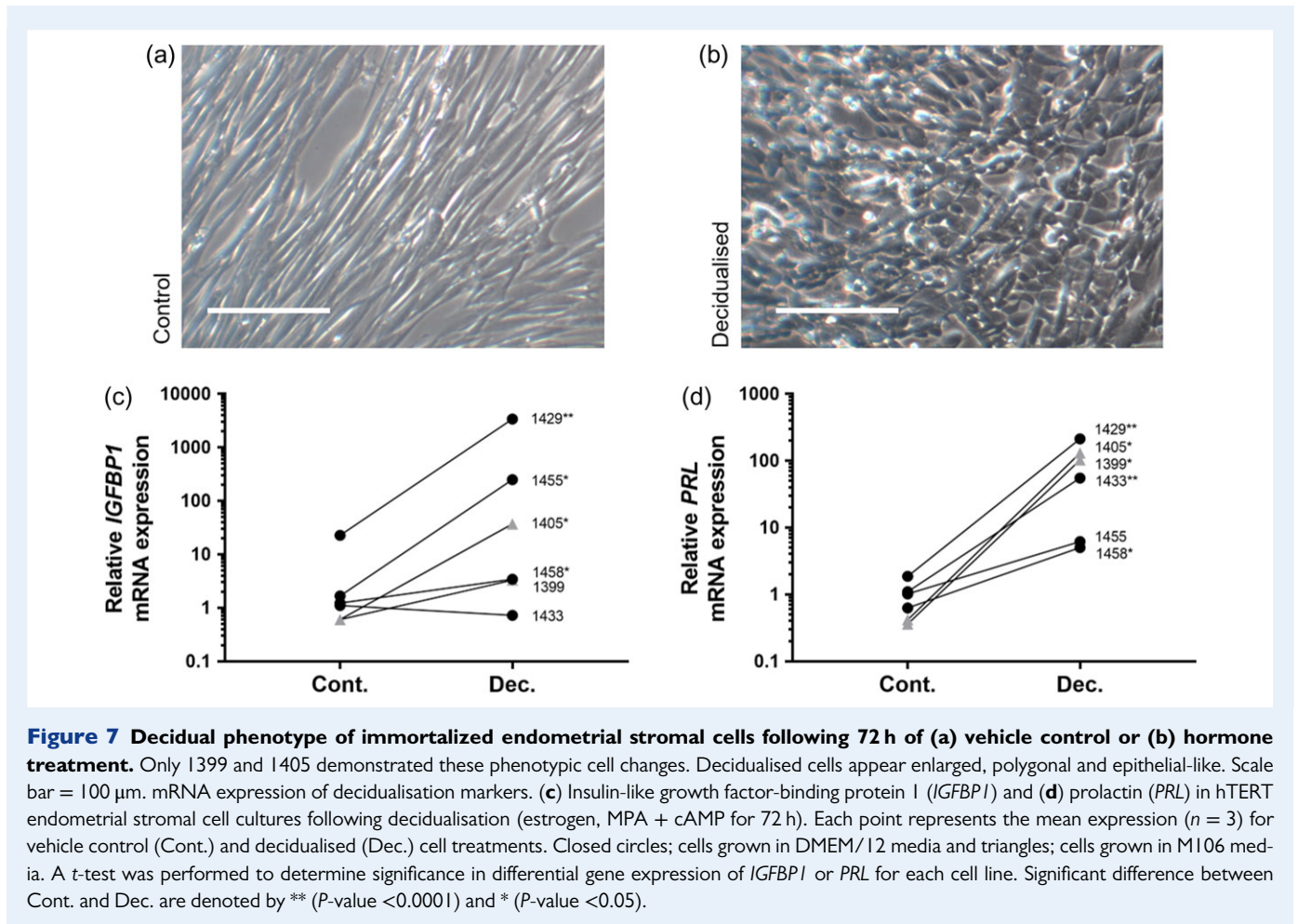

Figure 6 Varying cell proliferation of hTERT endometrial stromal cell cultures. (a) Doubling time of the T HESC cell line and 1399, 1405, 1429, 1433, 1455 and 1458. ^Cells grown in M106 medium prior to the assay. Doubling time was calculated on Day 3. (b) Proliferation measured over 6 days for the same cultures. Black circles; cells grown in DMEM/12 media, open circles; T HESC and grey triangles; cells grown in M106 media. (c–j) Proliferation fold change shown for Day 1 and Day 3 in response to hormone treatments: estradiol (E), medroxyprogesterone acetate (MPA) and E + MPA for the T HESC cell line and 1399, 1405, 1429, 1433, 1455 and 1458 hTERT ESC lines. Fold change shown relative to vehicle (dashed line = 1.0). Significant difference between vehicle and hormone treatment is denoted by * (*P*-value < 0.05).

Figure 7 Decidual phenotype of immortalized endometrial stromal cells following 72 h of (a) vehicle control or (b) hormone treatment. Only 1399 and 1405 demonstrated these phenotypic cell changes. Decidualised cells appear enlarged, polygonal and epithelial-like. Scale bar = 100 μm. mRNA expression of decidualisation markers. (c) Insulin-like growth factor-binding protein 1 (*IGFBP1*) and (d) prolactin (*PRL*) in hTERT endometrial stromal cell cultures following decidualisation (estrogen, MPA + cAMP for 72 h). Each point represents the mean expression (*n* = 3) for vehicle control (Cont.) and decidualised (Dec.) cell treatments. Closed circles; cells grown in DMEM/12 media and triangles; cells grown in M106 media. A *t*-test was performed to determine significance in differential gene expression of *IGFBP1* or *PRL* for each cell line. Significant difference between Cont. and Dec. are denoted by ** (*P*-value <0.0001) and * (*P*-value <0.05).

Inflammatory response of hTERT immortalized endometrial stromal cell lines

The hTERT hESC lines were exposed to increasing doses of LPS to determine if they would respond to an inflammatory stimulus. Overall, the new hTERT lines and T HESC exhibited a dose dependent linear increase to LPS when examining pro-inflammatory *CXCL8* and *IL1B* mRNA expression (Fig. 8a and b). There was also a dose dependent increase in *CXCL5* mRNA in response to LPS in 1433, 1405, 1455 and 1399 cell lines, but not the T HESC or the remaining hTERT hESC lines (Fig. 8c). There was little change in *NFKB1* expression in response to LPS stimuli (Fig. 8d). Most of the hTERT cell lines exhibited higher levels of *CXCL8*, *IL1B*, *CXCL5* and *NFKB1* mRNA expression relative to the T HESC line in the presence of vehicle and in response to LPS (Fig. 8a–d).

Discussion

Cultures of hESC are integral tools for laboratory-based investigations into the cellular mechanisms associated with endometriosis. To overcome the limitations of reduced life span of primary cell cultures and a lack of diversity of available commercial cell lines, we have generated our own hTERT immortalized hESC lines. The immortalized hESC maintained their fibroblast-like morphology and endometriosis-risk

genotype. Furthermore, the new hESC lines functioned like primary cultures showing positive but diverse responses to exogenous hormone and inflammatory stimuli, while maintaining significant hormone receptor expression levels. We have grown immortalized cultures beyond passage 20 and can report that they have retained endometrial stromal cell phenotype as described above. These immortalized cell lines have been genetically characterized and will facilitate future *in vitro* studies of endometriosis (and other reproductive traits) while recognizing the importance of genotype. Therefore, we have described and validated the use of hTERT immortalization of hESC with different endometriosis risk genotypes; this practice should be employed by other research groups to improve the quality and impact of laboratory-based cell research.

One of the primary aims of this work was to increase the genotypic diversity of hESC lines, with an emphasis on known endometriosis RAs. The SNP (rs12037376) with the largest effect size on endometriosis risk is at the *WNT4* (Wnt Family Member 4) 1p36.12 locus (*P*-value 8.87×10^{-17}) (Sapkota *et al.*, 2017). SNPs in this region demonstrate significant eQTL in whole blood and endometrium, decreasing the expression of a long intergenic non-protein coding RNA 339 (*LINC00339*), and include rs3820282, rs56318008, rs55938609, rs12037376 and rs7521902 (Powell *et al.*, 2016). In contrast, rs12061255 (minor allele) increased the expression of *LINC00339* in endometrium (Powell *et al.*, 2016). One patient was homozygous RA
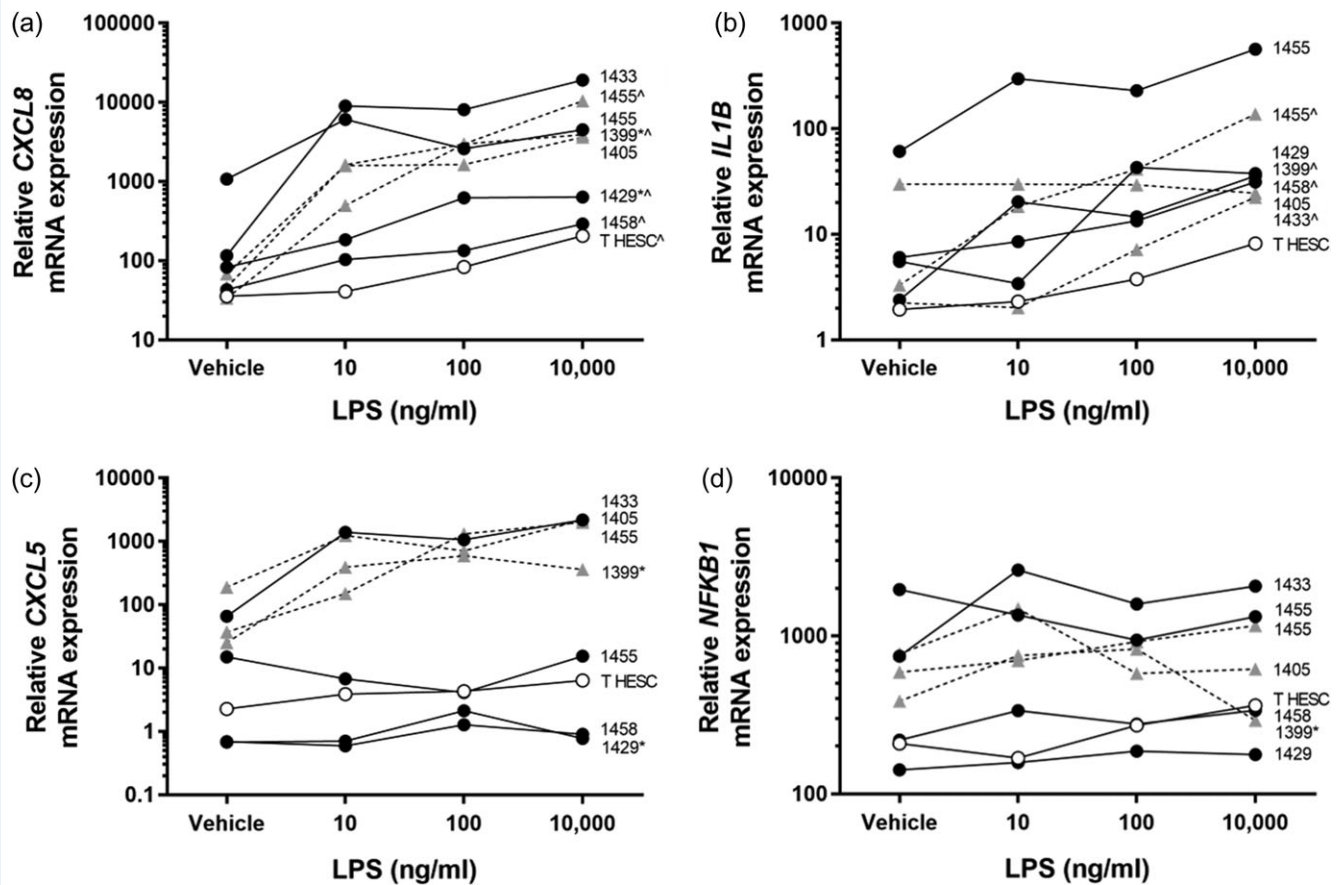
Figure 8 mRNA expression of inflammatory genes *CXCL8*, *IL1B*, *CXCL5* and *NFKB1* in hTERT endometrial stromal cultures and T HESC in response to 24 h lipopolysaccharide (LPS) treatment. Closed circles; cells grown in DMEM/12 media, open circles; T HESC and grey triangles; cells grown in M106 media. *Significantly different between vehicle and 100 ng/ml LPS (*P*-value <0.05). ^Significantly different between vehicle and 10 000 ng/ml LPS (*P*-value <0.05).

for five risk SNPs in the 1p36.12 region (including rs12037376 seen in Table II) and was also homozygous RA for 7 out of 14 key SNPs described in Table II (including the *GREB1*, *VEZT* and *7p15.2* loci); thus, we have generated a hTERT hESC line which is homozygous for multiple different RAs or key SNPs with genome wide significance for endometriosis (Sapkota *et al.*, 2017). While we have demonstrated that *LINC00339* is present in our cell lines, future work is necessary to perform *in vitro* assays comparing RA to OA hESC lines to determine functional cellular differences as a consequence of altered genotype. Furthermore and irrespective of endometriosis genetic risk loci, the genetic diversity displayed amongst the 13 cells lines generated in this study means that cell-based research will more closely reflect the patient to patient variability seen in women with endometriosis.

Endometriosis is an estrogen-dependent disorder and there is also evidence for a role of progesterone-resistance with the disease (Giudice, 2010). Therefore, the ability to appropriately respond to steroid hormones is a desirable feature of the new immortalized cell lines, particularly if they are to be valuable for future gynaecological research. We confirmed that introduction of hTERT had no significant effect on the median expression levels of estrogen and PRs or hormone responsiveness, with cell lines demonstrating a variable ability to

proliferate/regress and decidualise in response to hormonal stimuli. Inflammation is also an inherent response of the human endometrium and is integral to reproductive events including menstruation and implantation. Part of the endometrial stromal cell response to an inflammatory insult includes upregulated transcription of inflammatory response genes (e.g. transcription factors and cytokines). As expected, LPS-exposed hTERT hESC demonstrated a dose-dependent increase in expression of some pro-inflammatory cytokine genes, however, we did not see the expected increase in *NFKB1* (or *RelA* [unpublished data]). More detailed investigation of the NFκB transcription factor cascade (IKK complex, IκB and adaptor proteins like MyD88), and the LPS/TLR4 signal transduction pathway overall, may uncover more components involved in inflammation in our immortalized hESCs. The immortalized hESC lines developed in our laboratory display fundamental hormone- and inflammatory-response characteristics akin to endometrial stromal cells *in vivo*. It is important to highlight the diversity of these responses observed in our cell lines; which reflects normal variability and is a strength of our immortalized stromal cell model.

Others groups have overcome the limitations of primary culture by immortalizing hESC using various methods including simian virus 40 large T antigen (SV40) (Merviel *et al.*, 1995; Chapdelaine *et al.*, 2006;

Tamura *et al.*, 2007; Krishnaswamy *et al.*, 2009) and hTERT (Barbier *et al.*, 2005; Samalecos *et al.*, 2009; Yuhki *et al.*, 2011), including the only commercially available immortalized hESC line (Krikun *et al.*, 2004). We chose to utilize hTERT as our method of immortalization due to a high level of karyotype conservation following hTERT-immortalization compared to SV40 transfections in mammary cells (Toouli *et al.*, 2002). A recent advancement in gene editing, CRISPR/Cas9, has been employed in the cancer and cholesterol streams to specifically manipulate RAs (Coggins *et al.*, 2017; Davis *et al.*, 2018); this approach may provide more sensitive and higher-throughput methods for studying endometriosis risk genotypes in the future. Our cell lines demonstrated variability in the traits we examined including cell proliferation, hormone receptor expression and responsiveness to hormones and inflammation. These variations were highly reflective of normal patient-to-patient variability. With the genotype of each of our cell lines known, we can now commence functional assays and analyses of endometrial stromal cells that possess homozygous-risk or other alleles. Like *in vitro* cultures, rodent models of endometriosis have their limitations, as they do not completely recapitulate the human disease (Bruner-Tran *et al.*, 2018). Moving forward, *in vivo* rodent xenograft models using human immortalized endometrial cells carrying endometriosis risk genoytpes may be important for understanding the complex cooperative roles of genetics and the environment. Importantly, we have shown that the process of immortalization did not alter the SNP genotype of the selected hESC, therefore the endometriosis-risk genotypes were maintained. To the best of our knowledge, we are the first group to generate several hTERT immortalized hESC cell lines with endometriosis risk genotypes reflective of the varied genotypic backgrounds of individual patients.

The priority in the development of these cell lines was to generate a diverse set of hESC lines carrying important endometriosis-RAs. However, their utility goes beyond the setting of endometriosis research and the associated genetic implications, especially given the diversity of the cell lines reproducing true patient-to-patient variability. These immortalized cell lines will be valuable research tools for many different endometrial pathologies (e.g. recurrent miscarriage and implantation failure). In conclusion, our new set of well characterized immortalized hESCs allows us to undertake *in vitro* experimentation with a level of genetic and phenotypic diversity not previously available.

Acknowledgements

Authors' roles

S.J.H.-C., E.M.C., J.F.D., J.N.F., M.L.C. and P.P. performed the experiments. S.J.H.-C. wrote the article. S.J.H.-C., E.M.C., J.F.D., J.N.F., M.L. C., S.M., P.P., M.H., G.W.M., J.E.G. and P.A.W.R. contributed to the design of the study, analysis and interpretation of the data, drafting of the article and final approval of the version to be published.

Funding

Conflict of interest

All authors declare that they have no conflict of interest.

References

Barbier C, Becker K, Troester M, Kaufman D. Expression of exogenous human telomerase in cultures of endometrial stromal cells does not alter their hormone responsiveness. *Biol Reprod* 2005;**73**:106–114.

Bruner-Tran KL, Mokshagundam S, Herington JL, Ding T, Osteen KG. Rodent models of experimental endometriosis: identifying mechanisms of disease and therapeutic targets. *Curr Womens Health Rev* 2018;**14**: 173–188.

Chapdelaine P, Kang J, Boucher Kovalik S, Caron N, Tremblay J, Fortier M. Decidualization and maintenance of a functional prostaglandin system in human endometrial cell lines following transformation with SV40 large T antigen. *Mol Hum Reprod* 2006;**12**:309–319.

Coggins N, Stultz J, O'geen H, Carvajal Carmona L, Segal D. Methods for scarless, selection-free generation of human cells and allele-specific functional analysis of disease-associated SNPs and variants of uncertain significance. *Sci Rep* 2017;**7**:15044.

Davis J, Vadlamudi S, Roman T, Zeynalzadeh M, Iyengar A, Mohlke K. Enhancer deletion and allelic effects define a regulatory molecular mechanism at the VLDLR cholesterol GWAS locus. *Hum Mol Genet* 2018. doi: 10.1093/hmg/ddy385. [Epub ahead of print].

Dimitriadis E, Robb L, Salamonsen LA. Interleukin 11 advances progesterone-induced decidualization of human endometrial stromal cells. *Mol Hum Reprod* 2002;**8**:636–643.

Dimitrov R, Timeva T, Kyurkchiev D, Stamenova M, Shterev A, Kostova P, Zlatkov V, Kehayov I, Kyurkchiev S. Characterization of clonogenic stromal cells isolated from human endometrium. *Reproduction* 2008;**135**: 551–558.

Fung JN, Girling JE, Lukowski SW, Sapkota Y, Wallace L, Holdsworth-Carson SJ, Henders AK, Healey M, Rogers PAW, Powell JE *et al.* The genetic regulation of transcription in human endometrial tissue. *Hum Reprod* 2017;**32**:893–904.

Fung J, Holdsworth-Carson S, Sapkota Y, Zhao Z, Jones L, Girling J, Paiva P, Healey M, Nyholt D, Rogers P *et al.* Functional evaluation of genetic variants associated with endometriosis near GREB1. *Hum Reprod* 2015a; **30**:1263–1275.

Fung JN, Mortlock S, Girling JE, Holdsworth-Carson SJ, Teh WT, Zhu Z, Lukowski SW, Mckinnon BD, Mcrae A, Yang J *et al.* Genetic regulation of disease risk and endometrial gene expression highlights potential target genes for endometriosis and polycystic ovarian syndrome. *Sci Rep* 2018;**8**:11424.

Fung JN, Rogers PA, Montgomery GW. Identifying the biological basis of GWAS hits for endometriosis. *Biol Reprod* 2015b;**92**:87.

Gibson G, Powell JE, Marigorta UM. Expression quantitative trait locus analysis for translational medicine. *Genome Med* 2015;**7**:60.

Giudice LC. Clinical practice. Endometriosis. *N Engl J Med* 2010;**362**: 2389–2398.

Holdsworth-Carson SJ, Fung JN, Luong HT, Sapkota Y, Bowdler LM, Wallace L, Teh WT, Powell JE, Girling JE, Healey M *et al*. Endometrial vezatin and its association with endometriosis risk. *Hum Reprod* 2016; **31**:999–1013.

Kommagani R, Szwarc MM, Vasquez YM, Peavey MC, Mazur EC, Gibbons WE, Lanz RB, Demayo FJ, Lydon JP. The promyelocytic leukemia zinc finger transcription factor is critical for human endometrial stromal cell decidualization. *PLoS Genet* 2016;**12**:e1005937.

Krikun G, Mor G, Alvero A, Guller S, Schatz F, Sapi E, Rahman M, Caze R, Qumsiyeh M, Lockwood CJ. A novel immortalized human endometrial stromal cell line with normal progestational response. *Endocrinology* 2004;**145**:2291–2296.

Krishnaswamy N, Chapdelaine P, Tremblay J, Fortier M. Development and characterization of a simian virus 40 immortalized bovine endometrial stromal cell line. *Endocrinology* 2009;**150**:485–491.

Luong HT, Painter JN, Shakhbazov K, Chapman B, Henders AK, Powell JE, Nyholt DR, Montgomery GW. Fine mapping of variants associated with endometriosis in the WNT4 region on chromosome 1p36. *Int J Mol Epidemiol Genet* 2013;**4**:193–206.

Medicine ASFR. Revised American Society for Reproductive Medicine classification of endometriosis: 1996. *Fertil Steril* 1997;**67**:817–821.

Merviel P, Degeorges A, Salat Baroux J, Calvo F. Normal human endometrial cells in culture: characterization and immortalization of epithelial and stromal cells by SV 40 large T antigen. *Biol Cell* 1995;**84**:187–193.

Powell JE, Fung JN, Shakhbazov K, Sapkota Y, Cloonan N, Hemani G, Hillman KM, Kaufmann S, Luong HT, Bowdler L *et al*. Endometriosis risk alleles at 1p36.12 act through inverse regulation of CDC42 and LINC00339. *Hum Mol Genet* 2016;**25**:5046–5058.

Rashidi N, Mirahmadian M, Jeddi-Tehrani M, Rezania S, Ghasemi J, Kazemnejad S, Mirzadegan E, Vafaei S, Kashanian M, Rasoulzadeh Z *et al*. Lipopolysaccharide- and lipoteichoic acid-mediated pro-inflammatory cytokine production and modulation of TLR2, TLR4 and MyD88 expression in human endometrial cells. *J Reprod Infertil* 2015; **16**:72–81.

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

Sampson JA. Heterotopic or misplaced endometrial tissue. *Am J Obstet Gynecol* 1925;**10**:649–664.

Sapkota Y, Steinthorsdottir V, Morris AP, Fassbender A, Rahmioglu N, De Vivo I, Buring JE, Zhang F, Edwards TL, Jones S *et al*. Meta-analysis identifies five novel loci associated with endometriosis highlighting key genes involved in hormone metabolism. *Nat Commun* 2017;**8**: 15539.

Tamura K, Yoshie M, Hara T, Isaka K, Kogo H. Involvement of stathmin in proliferation and differentiation of immortalized human endometrial stromal cells. *J Reprod Dev* 2007;**53**:525–533.

Toouli C, Huschtscha L, Neumann A, Noble J, Colgin L, Hukku B, Reddel R. Comparison of human mammary epithelial cells immortalized by simian virus 40 T-Antigen or by the telomerase catalytic subunit. *Oncogene* 2002;**21**:128–139.

Yuhki M, Kajitani T, Mizuno T, Aoki Y, Maruyama T. Establishment of an immortalized human endometrial stromal cell line with functional responses to ovarian stimuli. *Reprod Biol Endocrinol* 2011;**9**:104.