Evaluating the influence of progesterone concentration and time of exposure on in vitro endometrial decidualisation

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The authors declare that they have nothing to disclose.

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Highlights

- A high progesterone concentration is associated with improvement in the decidualisation process.
- The most functionally and morphologically active part of the decidualisation process occurred 7-9 days after progesterone exposure.
- According to a pathway enrichment analysis, inhibition of cell cycle progression is the major effect of progesterone on Human Endometrial Stromal Cells (HESC).

Abstract

This study aimed to evaluate the influence of progesterone (concentration and time of exposure) on endometrial decidualisation using an in vitro model cell line: Human Endometrial Stromal Cells (HESCs). HESCs exposed to progesterone (1 and 10 μM) had higher percentages of decidualised cells and higher expression of the decidual marker (Insulin Like Growth Factor Binding Protein 1 (IGFBP1)) compared with those exposed to (0.1 μM) . Among those HESCs cultured with 1 μM progesterone for 11 days, the highest rate of morphological differentiation (40-50%) occurred between days 7-9 and IGFBP1 peaked on day 7. The cell-cycle pathway was significantly down-regulated in HESCs exposed to at least 1 μM progesterone regardless of the incubation period. We conclude that exposure to high progesterone concentration for 7-9 days is essential to maximise the process of decidualisation.

Keywords: progesterone, endometrium, implantation window, decidualisation, human endometrial stromal cells, microarray.

1. Introduction

The endometrium is a highly dynamic tissue undergoing extensive growth and repeated regeneration in a cyclical manner (Henriet, Chevronnay and Marbaix, 2012,Petracco, Kong, Grechukhina et al., 2012). The endometrium undergoes a cycle of dislodgment, proliferation, and secretory differentiation regulated by the sequentially timed interplay of circulating steroid hormones. Oestrogen is necessary for inducing expression of progesterone receptors (PR) and increasing endometrial thickness in the proliferative phase (Hamilton, Arao and Korach, 2014,Koos, 2011). Progesterone plays an important role in the secretory transformation of the endometrium and receptivity to the implanting embryo (Okada, Tsuzuki and Murata, 2018). Decidualisation occurs during the implantation window during the secretory phase corresponding to the physiological peak in progesterone concentrations in the menstrual cycle (Reed and Carr, 2015).

Decidualisation is a progesterone-dependent differentiation process of endometrial stromal cells characterised by differentiation from elongated fibroblast-like mesenchymal cells in the stromal compartment into rounded epithelioid-like cells during the secretory phase (Coulam, 2016). The peak of morphological differentiation during the secretory phase is observed during the implantation window (Luesley and Kilby, 2016). Decidualisation process is a prerequisite for successful embryo implantation and provides nutritional support for the implanting blastocyst (Su and Fazleabas, 2015). The decidualisation process provides both a protective function to limit trophoblast invasion and a supportive role in placentation by secreting growth factors and cytokines that assist remodel the implantation site and the maternal vasculature to promote embryo implantation and growth (Ramathal, Bagchi, Taylor et al., 2010). Insulin Like Growth Factor Binding Protein 1 (IGFBP1) is a major product of decidualised endometrial stromal cells and ~~may regulate endometrial differentiation and implantation~~ thus it is considered a preferred molecular marker of human decidualisation (Tseng, Gao, Chen et al., 1992,Gellersen and Brosens, 2003). Furthermore, IGFBP-1 produced by decidualised endometrium can directly stimulate endometrial stromal cell decidualisation by interacting with α5β1 integrin on the surface of endometrial stromal cells (Matsumoto, Sakai and Iwashita, 2008). It has been shown that defective decidualised stromal cells secrete lower IGFBP1

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(Garrido-Gomez, Dominguez, Quiñonero et al., 2017). With this concept, although there is no cut-off level of IGFBP1 and no randomised controlled trial indicating lower levels of IGFBP1 is associated with impaired decidualisation; lower expression of IGFBP1 compared with the reference or control samples is commonly considered as possible indicator of poor decidualisation in the scientific literature (Vinketova, Mourdjeva and Oreshkova, 2016). Impairment of decidualisation is associated with various reproductive disorders, such as infertility, recurrent miscarriages, and uteroplacental disorders (Garrido-Gomez et al., 2017,Wu, Kimura, Zheng et al., 2017,Cha, Sun and Dey, 2012).

Progesterone supports endometrial receptivity and decidualisation. Abnormally low serum progesterone concentrations during the implantation window have been shown to be associated with poorer pregnancy rates in both natural cycles (Jordan, Craig, Clifton et al., 1994,Radwanska, Hammond and Smith, 1981,Hull, Savage, Bromham et al., 1982) and frozen-thawed cycles (Basnayake, Volovsky, Rombauts et al., 2018,Labarta, Mariani, Holtmann et al., 2017,Cédrin-Durnerin, Isnard, Mahdjoub et al., 2019). In frozen-thawed cycles, the number of days of progesterone exposure prior to embryo transfer is based on the stage of the frozen embryos to be transferred. Too early or delayed progesterone administration results in a poorer pregnancy rate (Sharma and Majumdar, 2016). Those finding suggests that both progesterone concentrations and timings of exposure are critical factors to achieve implantation.

Decidualisation can be artificially induced using progesterone supplementation. In vitro studies using artificial decidualisation models have allowed us to improve our insights into the effect of progesterone on the process of decidualisation. The in vitro model offers a controlled environment to define the particular genes differentially expressed under the influence of progesterone with stratified datasets based on time and concentration and it can be used to identify progesterone-associated pathways. Human Endometrial Stromal Cells (HESC) respond to progesterone stimulation and show the morphological pattern and biochemical endpoints of decidualisation (Krikun, Mor, Alvero et al., 2004). These cells are karyotypically, morphologically, and phenotypically similar to the primary parent cells retrieved from the human uterus. The consistency and reproducibility of results is also the

major advantage of HESC culture, as HESC can be cultured and tested repeatedly. Importantly, the exact progesterone concentration and timing of exposure can be controlled precisely under in vitro conditions, Therefore in vitro models of artificial decidualisation are an excellent model to study the effect of progesterone on decidualisation and the potential for implantation (Krikun et al., 2004).

Defective decidualisation has been found as a root cause for implantation failure and subsequent early embryo miscarriage (Gellersen and Brosens, 2014,Kommagani, Szwarc, Vasquez et al., 2016). If we know the optimal progesterone concentration and duration of progesterone exposure that maximises decidulisation process, it could guide how to improve this process by adjusting progesterone administration. We aim to investigate the impact of different progesterone concentrations and different timings of progesterone exposure on the decidualised endometrium using endometrial morphology, decidual markers, and global gene expression using microarray-based technology.

2. Objectives

To study endometrial HESC gene expression profiles and endometrial morphology following in vitro incubation for 3, 7, and 11 days with increasing concentrations of progesterone (0.1, 1, and 10µM)).

3. Methods

3.1 Characterisation and culture of HESCs

This study consisted of morphological and functional assessments of HESCs. HESCs decidualisation in response to progesterone was assessed morphologically (change from elongated to rounded cells) and by expression of a decidualisation marker (IGFBP1). The day of peak expression of IGFBP1 (day 7) was used as a reference to compare the whole transcriptomic profile of HESCs between different progesterone concentrations and between different incubation periods using microarray (Figure 1).

The human endometrial stromal cells (HESCs; ATCC CRL-4003; American Type Culture Collection Co., Virginia, USA) were cultured in Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM/F12, Sigma, Poole, UK) supplemented with 1% ITS+ Premix (Thermo Fisher Scientific,

Loughborough, UK), 500 ng/mL puromycin (Thermo Fisher Scientific, Loughborough, UK), 10% charcoal/dextran treated fetal bovine serum (HyClone, Thermo Fisher Scientific, Loughborough, UK) at 37°C in a humidified chamber with 5% CO2. The medium was changed every 48 hours (Huang, Yu, Li et al., 2017).

To induce in vitro decidualisation, HESCs were incubated in DMEM/F12 medium supplemented with 2% CS-FBS, 10 nM β-oestradiol, 0.5 mM 8-Bromoadenosine 3′,5′-cyclic monophosphate sodium salt (8-Br-cAMP, Sigma, Poole, UK) and increasing concentrations of progesterone (P4, 0.1, 1 and 10 μM; 4-pregnene-3,20-dione; Sigma, Poole, UK) (Li, Kang, Qiao et al., 2017). The progesterone concentration of 1 μM is the standard concentration of HESC decidualisation in vitro (Krikun et al., 2004). These doses were based on a preliminary dose-response experiment (data not presented), showing that doses above 30 uM, decreased survival within 48 hours. A dose of 20 uM decreased survival in some replicates after 96 hours. A dose of 10 uM did not decrease cell survival in a period of 11 days, this concentration was used as the highest concentration in this experiment. A light microscope was used to differentiate rounded (decidualised) cells from elongated stromal cells. The percentages of decidualised HESCs were visually estimated in 10 fields at 10X magnification and expressed as the average percentage from the total cells in each field on days 3, 5, 7, 9, and 11.

Moreover, IGFBP1 expression, a marker of decidualisation and endometrial receptivity (Tseng et al., 1992,Gellersen and Brosens, 2003), was assessed by PCR following incubation for 3, 5, 7, 9, and 11 days, to determine the incubation time required to induce decidualisation in HESC under increasing concentrations of progesterone, similar to morphological assessment. The day of peak expression of IGFBP1 was used as a reference for microarray analysis.

3.2 Determining whole transcriptomic profile by microarray

The experimental design for the microarray experiment is presented in Figure 1. To determine the effect of different progesterone concentrations on endometrial transcriptomics, the gene expression of HESCs treated with 0.1, 1, and 10 µM progesterone was analysed on day 7 (peak IGFBP1 based on our findings (Figure 3)). To evaluate the effect of the incubation period, the global gene expression of HESCs treated with a standard concentration of progesterone (1 µM) was studied on days 3, 7, and 11 of incubation (Li et al., 2017). The standard progesterone concentration of 1 µM was taken from the standard protocol of HESC decidualisation in vitro (Krikun et al., 2004). Differentially expressed genes with fold changes were obtained from the comparison of gene expression between HESCs exposed to progesterone in a particular concentration and incubation period (intervention groups) and HESCs cultured in the medium without progesterone for the same period (control groups). On a corresponding day, media was collected and lysis buffer added (150 µL/well), followed by scrapping to remove cells. Lysis buffer containing the cells was pipetted into an Eppendorf tube (1.5ml) and stored at -80°C until RNA extraction. Each treatment was run in triplicate.

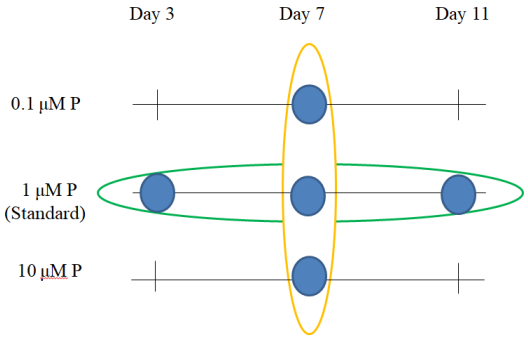


Figure 1: Diagram representing HESCs treated with increasing progesterone concentrations (0.1 µM, 1 µM, and 10 µM) and different incubation periods (3, 7, 11 days).

3.2.1 Transcriptome profiling with Affymetrix GeneChip

The whole-genome transcriptome analysis of HESCs treated with different progesterone concentrations at specific incubation periods was conducted at the Nottingham Arabidopsis Stock Centre (NASC). The RNA concentration and quality were assessed using the Agilent 2100 Bioanalyzer (Agilent Technlogies Inc., Palo Alto, CA, USA) and the RNA 600 Nano Kit (Caliper Life Sciences, Mountain View, CA, USA). Samples with a minimum RNA concentration of 100 ng/µl and RNA Integrity Number (RIN) ≥ 8 were used for gene expression analysis. Single-stranded complementary DNA was prepared from 200 ng of total RNA as per the GeneChipTM WT PLUS Reagent Kit (Applied Biosystems and Affymetrix, Loughborough, UK). Total RNA was first converted to cDNA, followed by in vitro transcription to make cRNA. Single-stranded cDNA was synthesized, end-labeled, and hybridized for 16 h at 45°C to Clariom™ S Assay arrays (Thermo Fisher Scientific, Loughborough, UK).

3.2.2 Expression array analysis

Gene expression data and pathway analysis were used to compare endometrial response between HESC exposed to different progesterone concentrations and incubation periods. Gene expression data were analysed using Partek Genomics Suite 6.6 software (Partek Incorporated). The raw CEL files were normalized using the RMA background correction with quantile normalization, log base 2 transformation, and mean probe-set summarization with adjustment for GC content. Differentially expressed genes (DEG) were identified by a two-way ANOVA. DEGs were considered significant if p-value with FDR was ≤ 0.05 and fold change of >1.5 or <-1.5.

Venn diagrams were generated to display the number of overlapping genes among three different progesterone concentrations and three different incubation periods. Gene Ontology (GO) and pathways enrichment analyses were carried out by using the webgestalt web tool (http://www.webgestalt.org/) (Liao, Wang, Jaehnig et al., 2019). The microarray data has been deposited in the NBCI Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/geo), accession number: GSE146777. Microarray validation was carried out by RT-PCR on four significantly

upregulated (IGFBP1, SPP1, GPX3, and MAOA) and four downregulated genes (IFIT1, MOXD1, CDK15, and CDC20) in all progesterone concentrations on days 3, 7, and 11.

3.2.3 RNA preparation and PCR

Total RNA was extracted using the RNeasy Mini Kit (QIAGEN, Manschester, UK) following the manufacturer's indications. Quantification of RNA was assessed using a Nanodrop Spectrophotometers (Thermo Fisher Scientific, Loughborough, UK). After extraction, a High-Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Catalog number: 4374967, Thermo Fisher Scientific, Loughborough, UK) was used to prepare the cDNA template. A mixture of 1.5 µL of cDNA, 0.75 µL of TaqMan Assays, 7.5 µL TaqMan fast universal mastermix; and 5.25 µL RNAse-Free Water was prepared to perform PCR. To determine the expression of the genes of interest, the following Taqman primers (Thermo Fisher Scientific, Loughborough, UK) were used as appropriate: SPP1 (Hs00959010_m1), GPX3 (Hs00173566_m1), MAOA (Hs00165140_m1), IGFBP1 (Hs00236877_m1), IFIT1 (Hs03027069_s1), MOXD1 (Hs01026922_m1), CDK15 (Hs00287045_m1), and CDC20 (Hs00961704_g1). All PCR reactions were performed in triplicate. The PCR was performed with a heating step at 95°C for 20 minutes and then cycled 40 times at 95°C for 3 seconds followed by 60°C for 30 seconds on the ABI 7500 fast real-time PCR system (Thermo Fisher Scientific, Loughborough, UK). A housekeeping gene expression (18S, Hs99999901_s1), was used to normalise the gene expression data. The gene expression was expressed as the n-fold difference relative to the control.

3.3 Statistical analysis

Statistical analysis was performed using SPSS (v26; IBM; Portsmouth, UK). A two-way ANOVA was conducted to compare the effect of progesterone concentrations and incubation periods on decidualisation. A one-way ANOVA was performed to examine the effect of three different progeterone on the number of decidualised cells. Student's t-test was used to determine the difference

of expression of selected genes between control and experimental groups for microarray validation. A p-value < 0.05 was considered to be statistically significant.

4 Results

4.1 Decidualisation in vitro

The morphology of non-decidualised and decidualised HESCs are shown in Figure 2A and 2B, respectively. There was a difference in the mean percentage of decidualised cells between progesterone concentrations ($p < 0.001$) and incubation periods ($p < 0.001$), with an interaction between these ($p < 0.001$) (Figure 2). Decidualised cells were present on days 3 and 5, but the percentage increased from day 7 onwards, peaking on day 11. This was similar across all progesterone concentrations (0.1, 1, 10 μM; Figure 2C). On days 7, 9, and 11, higher progesterone concentrations allowed higher accumulative percentages of decidualised cells (Figure 2C). The findings suggest that higher progesterone concentration and a longer incubation period increase the percentage of decidualised cells. Furthermore, there was a statistically significant difference between the three concentrations on days 7, 9, and 11 (Figure 2C). Therefore, the number of decidualised cells cultured with 10 uM are higher than those cultured with 0.1 and 1 uM. A concentration of 1 uM progesterone resulted in a higher number of decidualised cells than 0.1 uM progesterone.

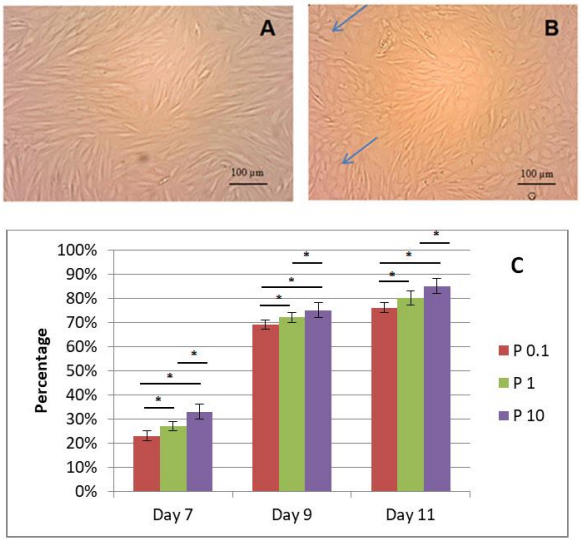


Figure 2: Non-decidualised cells present a fusiform shape (2A), while decidualised cells (arrows) are characterised by a round shape (2B), 10 x magnification. The accumulative percentages of decidualised cells following an incubation period of 7, 9, and 11 days with progesterone are shown in Figure 2C, the percentage was calculated from the average percentages of 10 fields. There was an effect of progesterone (P) concentration ($p < 0.001$), incubation time ($p < 0.001$) and its interaction ($p < 0.001$). * = significant difference between groups, $p < 0.001$ for all comparison.

4.2 IGFBP1 expression

The IGFBP1 expression increased as progesterone concentrations increased, the highest concentration (10 µM) showed the highest ($p < 0.001$) expression of IGFBP1 (Figure 3). The IGFBP1 expression peaked on day 7, regardless of progesterone concentration (Figure 3).

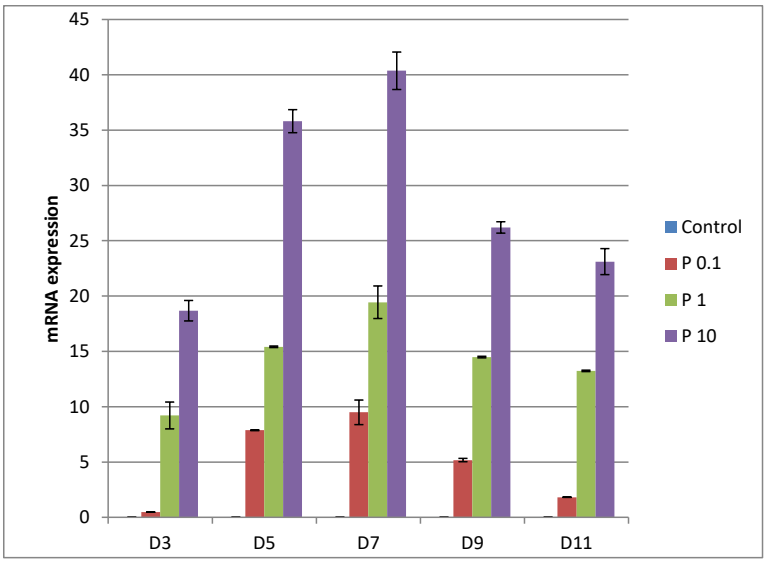


Figure 3: Mean (±s.e.m) IGFBP1 expression in human endothelial stromal cells (HESC) cultured in media containing increasing progesterone concentrations (0.1 μM, red bars; 1 μM, green bars; and, 10 μM, purple bars) for 11 days. There was an effect of progesterone (P) concentration ($p < 0.001$), incubation time ($p < 0.001$), however its interaction was not statistically significant ($p = 0.15$).

4.3 Pathway enrichment analysis

The results of pathway enrichment analysis were unique depending on different progesterone concentrations/incubation periods (Figure 4). The pathway related to cell cycle was significantly down-regulated in most experimental conditions, except when cells were incubated with 0.1 μM progesterone on day 7 (Figure 4). A full list of differentially expressed genes in each condition was provided in Supplementary 1.

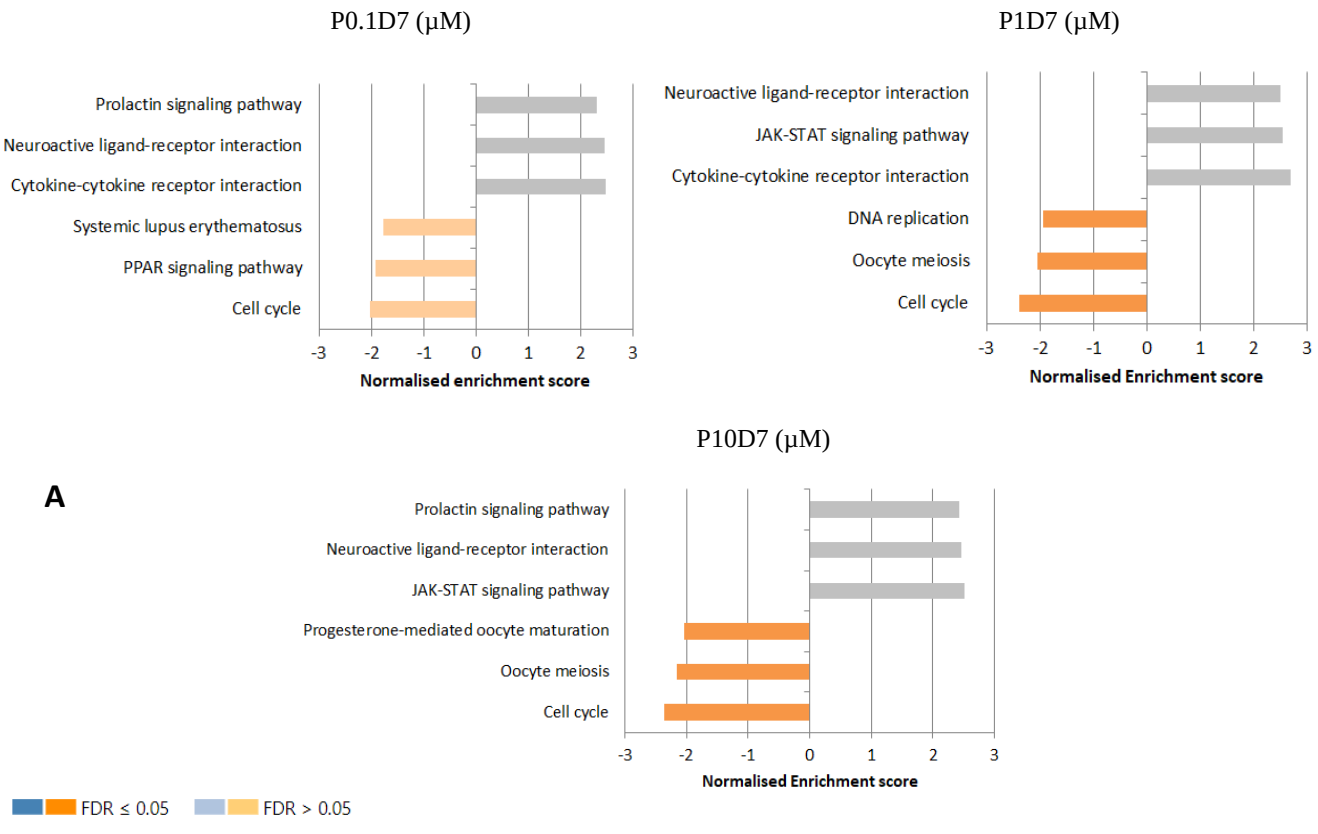
To determine the gene expression trend of cell cycle-regulated genes, the overlapping cell cycle-regulated genes between different concentrations (16 genes) and incubation periods (14 genes) were extracted (Figure 5) and the change of their gene expression was evaluated in Figure 6. With HESC

exposed to 10 µM progesterone, genes that are associated with cell-cycle regulation had higher negative fold change compared with HESCs exposed to 1 µM (Figure 6). The majority of cell cycle-regulated genes (12/14 genes) were expressed the most on day 3 compared with days 7 and 11. A list of cell cycle-regulated genes with fold changes in each condition was provided in Supplementary 2.

We further examined the gene expression trend of decidualisation-related genes, as described by (Liu and Wang, 2015), between different concentrations and incubation periods. We found that four out of the 12 decidualisation-related genes were significantly up-regulated in all experimental conditions regardless of concentrations or incubation periods (PRL, VIM, IL1B, and FOXO1). The expression of these and IGFBP1 increased with increasing progesterone concentrations (Figure 7). The highest fold change was observed on day 3 and decreased thereafter (Figure 7). A list of decidualisation-regulated genes with fold changes in each condition was provided in Supplementary 3.

4.4 Microarray validation by RT-PCR

Microarray results were validated by RT–PCR. All genes selected showed a similar pattern to that observed in the microarray analysis (Supplementary 4).


P0.1D7 (µM)
Prolactin signaling pathway
Neuroactive ligand-receptor interaction
Cytokine-cytokine receptor interaction
Systemic lupus erythematosus
PPAR signaling pathway
Cell cycle
-3 -2 -1 0 1 2 3
Normalised enrichment score
P1D7 (µM)
Neuroactive ligand-receptor interaction
JAK-STAT signaling pathway
Cytokine-cytokine receptor interaction
DNA replication
Oocyte meiosis
Cell cycle
-3 -2 -1 0 1 2 3
Normalised Enrichment score
P10D7 (µM)
A
Prolactin signaling pathway
Neuroactive ligand-receptor interaction
JAK-STAT signaling pathway
Progesterone-mediated oocyte maturation
Oocyte meiosis
Cell cycle
-3 -2 -1 0 1 2 3
Normalised Enrichment score
FDR ≤ 0.05
FDR > 0.05

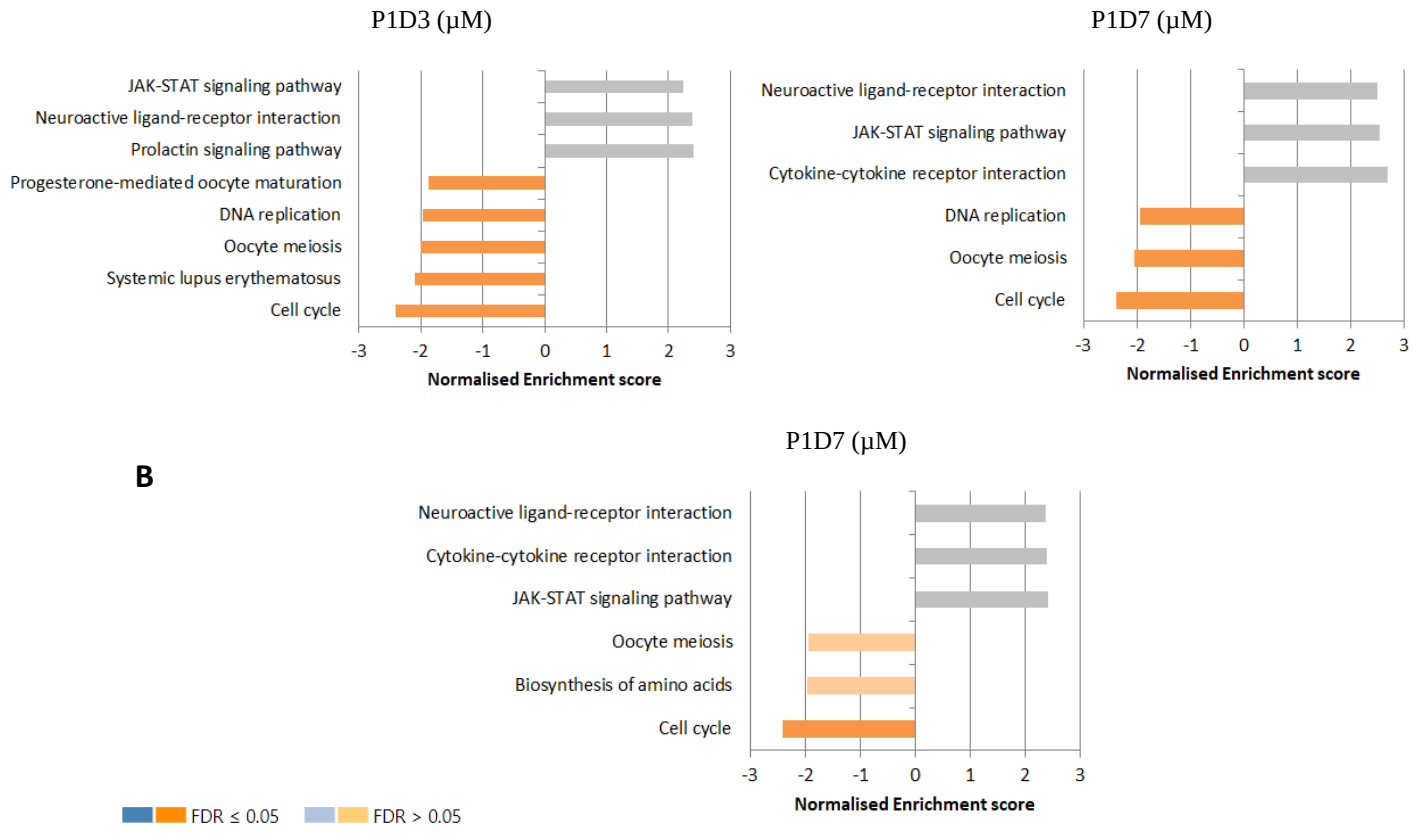
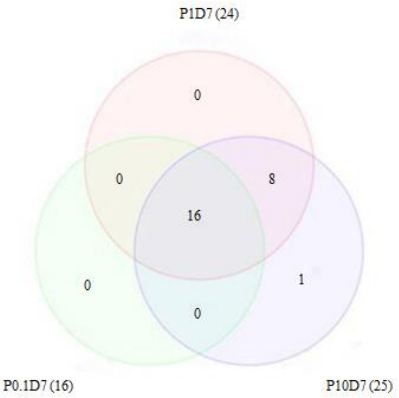


Figure 4: Bar charts representing pathway analysis between (A) increasing progesterone concentrations on day 7 (0.1, 1 and 10 µM) and (B) different incubation periods (3, 7 and 11 days). P=progesterone concentration and D=incubation day

A



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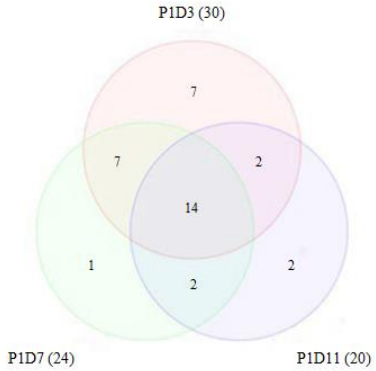
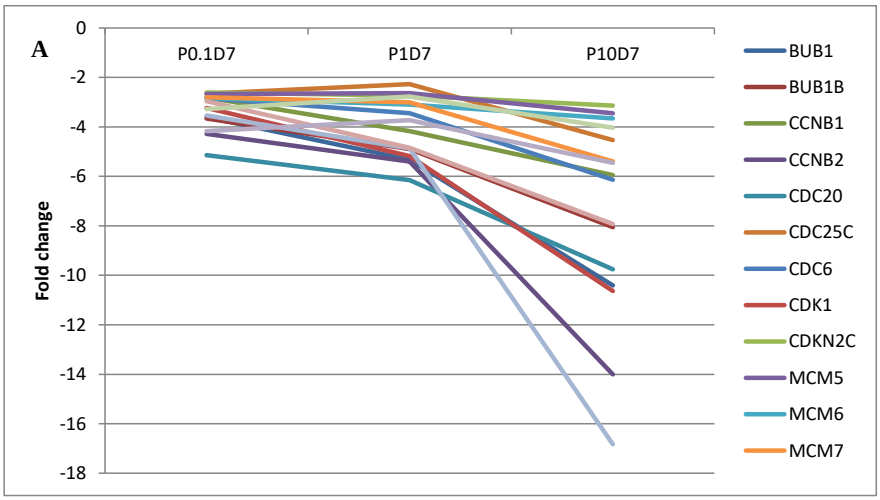


Figure 5: Overlap of cell cycle-regulated genes between different progesterone concentrations on day 7 (A; 0.1,1, and 10 µM) and on different incubation periods (B; 3, 7 and 11 days). P = progesterone concentration; D = incubation day.



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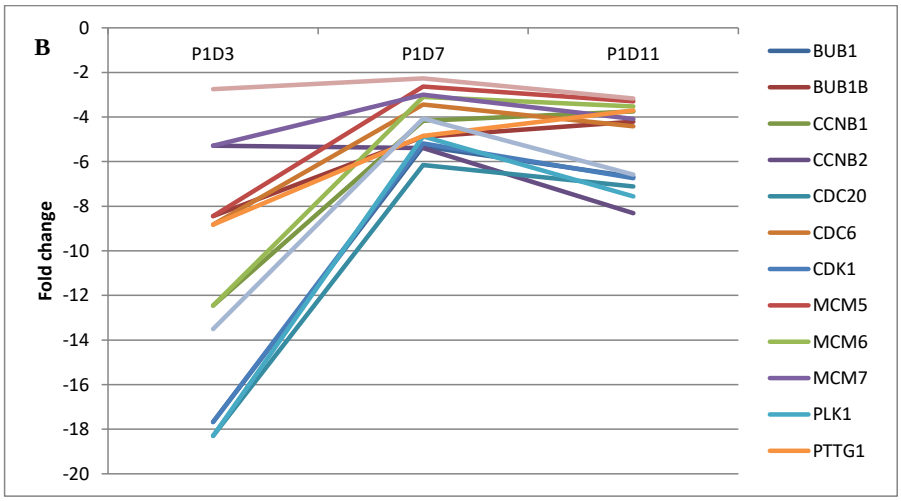

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318 **Figure 6**: Gene expression of overlapping genes involved in cell-cycle pathway between different
319 progesterone concentrations on day 7 (A; 0.1,1, and 10 µM); and the progesterone concentration of 1
320 µM between different incubation periods (B; 3, 7, and 11 days). P = progesterone concentration; D =
321 incubation day.

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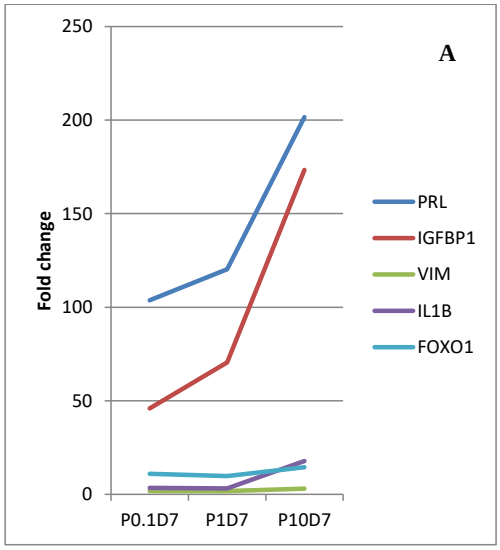

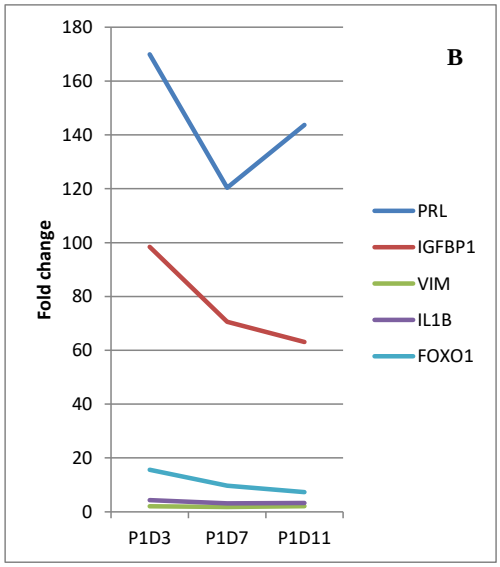

Figure 7: Gene expression of decidualisation-related genes between different progesterone concentrations (A; 0.1,1, and 10 µM); and the progesterone concentration of 1 µM between different incubation periods (B; 3, 7, and 11 days). P = progesterone concentration; D = incubation day.

5. Discussion

The results indicate that endometrial decidualisation is regulated by progesterone in a time and concentration-dependent fashion. Morphological differentiation was initially visualised on day 7. On day 7, 9, and 11, lower progesterone concentrations induced lower accumulative percentages of decidualised cells and lower expression of the decidualisation marker, IGFBP1. The findings suggest that lower progesterone concentration is potentially associated with impaired decidualisation. Several studies showed that women with low serum progesterone during the window of implantation had a significantly lower pregnancy rate in a natural cycle (Radwanska et al., 1981,Hull et al., 1982), fresh IVF cycle (Kaur, Naidu, Kumkum et al., 2018), IUI cycle (Labarta et al., 2017,Hansen, Eisenberg, Baker et al., 2018,Warne, Tredway, Schertz et al., 2011) and frozen-thawed embryo transfer cycle (Basnayake et al., 2018,Labarta et al., 2017). Therefore, adequate progesterone concentration is required during implantation to enhance the potential for implantation. Although the cut-off value is uncertain, a previous report from our group (Suthaporn, Jayaprakasan, Thornton et al., 2020) and other authors suggest a progesterone threshold ranging between 10 and 15 ng/ml. Our results suggest that lower progesterone concentration are potentially associated with compromised decidualisation, resulting in implantation failure.

In fact, spatio-temporal changes are required to transform the endometrium from a non-receptive to a receptive stage during the implantation window (Paria, Lim, Das et al., 2000,Aghajanova, Hamilton and Giudice, 2008). The peak of morphological differentiation during the secretory phase is observed during the implantation window following progesterone exposure for 7-9 days post-ovulation in a natural cycle (Luesley and Kilby, 2016). In this in vitro study, the expression of IGFBP1 peaked at day 7 and the greatest change of morphological differentiation occured between days 7 to 9 (40-50%) suggesting that the peak of decidual change in HESCs occurred 7-9 days following progesterone exposure which is consistent to the physiological findings observed in a natural cycle. Because ovulation typically occurs on day 14 of the menstrual cycle in women with a 28-day cycle, 7-9 days

after ovulation is approximately days 21-23 which is the period of implantation window (Harper, 1992,Wilcox, Baird and Weinberg, 1999), This suggests that the increased morphological differentiation could be associated with improved implantation potential. In a conception cycle, decidualisation spreads throughout the entire endometrium (Salamonsen and Evans, 2018). It is plausible that a greater number of decidualised cells will provide a larger area for blastocyst implantation, therefore higher progesterone concentration that allows a greater number of decidualised cells would be more favourable for implantation.

Decidualisation is characterised by stromal cell differentiation from fibroblast-like into larger and rounder decidual cells. This process is regulated by progesterone which promotes cell cycle arrest and inhibits proliferation before the cells start the differentiation process into decidual cells (Logan, Steiner, Ponnampalam et al., 2012). In this study, higher progesterone concentrations (1 and 10 µM) were associated with significant downregulation of the cell-cycle pathway, whereas the lowest concentration (0.1 µM) was not. In addition, higher progesterone concentrations activate higher expression of cell cycle-regulated genes (down-regulation) suggesting that greater progesterone concentration more effectively inhibits cell cycle progression, potentially resulting in a higher chance that cells stop cycling in order to further differentiate. Higher progesterone concentration also allows higher expression of decidualisation-regulated genes (PRL, IGFBP1, VIM, IL1B, and FOXO1; up-regulation). This suggests that after cell cycle progression is stopped, higher progesterone concentrations effectively promote stromal cell differentiation leading to higher chance of cells undergoing decidualisation. These mechanisms explain why the highest percentages of differentiated cells and the decidualisation marker, IGFBP1, were observed in HESCs exposed to the highest progesterone concentration.

The pathway involved in the cell cycle was the only pathway significantly down-regulated on all incubation periods (days 3, 7, and 11). In comparing the expression of cell cycle-regulated genes between those three conditions, it was found that the majority of those genes (12/14 genes) had the

highest expression (down-regulation) on day 3 suggesting that cell cycle progression was actively inhibited early on day 3, but increasingly inactivated on days 7 and 11. Interestingly, the highest expression of decidulisation-related genes (PRL, IGFBP1, VIM, IL1B, and FOXO1) also occurred on day 3 and subsequently reduced corresponding to the gene expression pattern of cell cycle-regulated genes. The findings indicate that the functional regulation of cell cycle arrest and differentiation was actively regulated in the early stage on day 3 after progesterone exposure consistent with the study by Takano et al. (Takano, Lu, Goto et al., 2007). In the study by Lucus et al., single-cell RNA sequencing (scRNA-seq) was used to assess temporal transcriptomic changes every 2 days for a period of 8 days in HESCs treated with progesterone, 5/12 decidualisation-regulated genes according to the study by (Liu and Wang, 2015), were significantly expressed on day 2 in response to progesterone (PGR, FOXO1, STAT3, VIM, and FN1) before morphological differentiation was observed, subsequently, those genes were less expressed in the later stage (Lucas, Vrljicak, Muter et al., 2020). Similarly, in this study, after extensive activation of decidualisation-related genes in an early stage on day 3 in response to progesterone, it takes a few days to generate morphological differentiation identified by light microscopy on day 7. After initiation of morphological differentiation, it is possible that extensive inhibition of cell cycle progression and activation of decidualisation-related genes is no longer needed, therefore the expression of those genes decreases on days 7 and 11.

The limitation of the study is that the conversion method of progesterone concentration in vitro to in vivo is not well-established. A concentration of 0.1, 1, and 10 uM can be converted to 31.5, 314.5, and 3144.6 ng/ml. The concentration of 0.1 uM (31.5 ng/ml) is close to physiologic serum progesterone during implantation. The higher concentration of 1 and 10 uM is supraphysiologic levels that are unlikely to occur in a natural cycle, yet 1 uM has been proposed as a standard concentration in vitro (Krikun et al., 2004). Therefore, this study provides informative details regarding the favourable trend of endometrial progesterone concentrations (high or low) and duration of progesterone exposure that maximises decidulisation process rather than point out an exact cut-off for progesterone

concentration. We believe that more research is needed to translate progesterone concentration from in vitro to in vivo study.

6. Conclusion

Decidualisation is modulated by progesterone exposure in a time and concentration-dependent manner. Exposure to below toxic (30 μM) but high (10 μM) progesterone concentration for 7-9 days is essential to maximise the process of decidualisation. Further pieces of evidence are required to determine whether higher decidualisation will translate into better clinical outcomes.

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Disclosure of interests

The authors declare that they do not have any conflict of interest.

Authors' contributions

WEM conceived this research project. SS, WEM, and JHM designed the study. SS, MC, and SM performed the experiments, processed the experimental data, and performed the analysis. SS, WEM, JHM, KFW, JGT, and KJ assisted in writing the paper.

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