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Circadian gene *PER1* senses progesterone signal during human endometrial decidualization

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Abstract

Progesterone is an important hormone for female reproduction; however, how the fluctuation of progesterone acts upon reproductive processes remains largely unknown. Mounting evidence indicates a pivotal role of the circadian clock in sensing hormone dynamics for homeostatic regulation of physiological functions. Therefore, we sought to determine whether clock genes respond to progesterone signaling in female reproductive system. In this study, we tested the hypothesis that the circadian system could respond to progesterone signaling during human endometrial decidual transformation. The expression of the circadian gene *PER1* increased immediately and remained elevated during human endometrial decidualization. The progesterone receptor activated *PER1* transcription by directly binding to its promoter from the onset of the stromal proliferation-differentiation transition. *PER1* knockout significantly downregulated the expression of some *PGR* target genes, and attenuated human endometrial decidual transformation by expediting *FOXO1* protein degradation. In conclusion, progesterone could control the female reproductive process through sustained feedback from the circadian gene *PER1*, which is probably involved to P_4 -PR signaling responsiveness in the initiation and maintenance of decidualization.

Key Words

- ▶ clock gene
- ▶ decidualization
- ▶ endometrium
- ▶ *PER1*
- ▶ progesterone receptor

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Introduction

Progesterone (P_4) is essential for regulating mammalian female reproduction. In the human menstrual cycle, P_4 secretion is minimal during the follicular phase and increases after ovulation, which plays a major role in preparing the uterus for embryo implantation and favors the establishment and maintenance of pregnancy. The effects of P_4 are mediated by P_4 binding with its cognate nuclear receptors, progesterone receptors (PRs, including PRA and PRB; both are encoded by the *PGR* gene), and the pleiotropic action of P_4 mainly relies on PR signaling

that occurs in a cell type-specific manner. Endometrial stromal cells, which are principal targets for P_4 in the uterus, undergo a dynamic morphological transformation process involving a proliferation-differentiation transition that is defined as decidualization (Gellersen & Brosens 2014) and is sustained by elevated P_4 levels in the luteal phase (Gellersen & Brosens 2003). It has been confirmed that compromised decidualization associated with aberrant P_4 -PR signaling could lead to a variety of infertility diseases, such as endometriosis (EMS).

However, our understanding of how P₄ modulates this process is still far from sufficient.

The circadian clock system exists to adapt to environmental signals such as hormonal dynamics, light–dark cycles, feeding and fasting, chronothermal signals or sleep–wake cycles (Papazyan *et al.* 2016). The rhythm pacemaker is generated within the suprachiasmatic nuclei (SCNs) of the hypothalamus and operates in both central and peripheral tissues, including the uterus (Mohawk *et al.* 2012, Miller & Takahashi 2013). The canonical circadian system consists of four genes (and their analogs): circadian locomotor output cycles kaput (*CLOCK*), brain muscle arnt-like 1 (*BMAL1*), period (*PER1*, *PER2*, and *PER3*) and cryptochrome (*CRY1* and *CRY2*). It has been validated that all these genes are expressed in the human, rat and mouse uterus, and some of them can be altered by ovarian hormones (Johnson *et al.* 2002, Nakamura *et al.* 2005, 2008, 2010, He *et al.* 2007, Hirata *et al.* 2009). *PER2* deficiency leads to flattened diurnal oscillation of all the core clock genes and to disorganized decidual responses in human endometrial stromal cells (HESCs) after continuous decidualization for 4 days by blocking the cell cycle, but whether clock genes mediate the responses of HESCs to P₄ signals remains unclear (Muter *et al.* 2015). In addition, knockout of *Pgr* results in decreased expression of many rhythm genes in the mouse uterus, e.g., *Per1* (Rubel *et al.* 2012); thus, we hypothesized that the regulatory mechanism controlling decidualization responses upon P₄ signaling might be mediated by other clock genes, such as *PER1*, rather than *PER2*.

To test this hypothesis, we investigated whether the expression of these clock genes changed at the beginning of the differentiation transition, a question that to our knowledge has not been addressed by previous studies. The conventional concept holds that the process of stromal cell differentiation commences with elevated intracellular cAMP levels and is then sustained by rising P₄ levels (Gellersen & Brosens 2003, 2014). cAMP signaling is also an intrinsic part of the pacemaker pathway for SCN cells and most peripheral cells throughout the body (Yagita & Okamura 2000, O'Neill *et al.* 2008, Zmrljak *et al.* 2013). Our data show that the immediate *PER1* response to P₄ signaling, but not to cAMP signaling, at the early stage of decidualization. To explore the regulatory effects of P₄ on clock genes, we used a CRISPR/Cas9 approach and a luciferase reporting system, through which we verified that the transcriptional activation of *PER1*, the core circadian gene, is directly regulated by PR beginning at the initiation of decidualization (Large & DeMayo 2012, Patel *et al.* 2015). Remarkably, *PER1* deficiency

leads to declined progesterone signal responsiveness and compromised decidualization at least partly by decreasing forkhead box O1 (FOXO1) protein stability.

Materials and methods

Patient selection and ethical approval

The study was performed based on the approval of the Medical Ethics Committee of Zhongnan Hospital of Wuhan University (2017061). Fifty-five women (age range 23–42 years) were recruited from the Reproductive Medicine Center of Zhongnan Hospital from January to December (2018). Written informed consent was obtained from all participants according to the guidelines of the Declaration of Helsinki. For analysis of the expression of *PER1* mRNA levels, a total of 46 biopsies were obtained from patients who had regular ovulatory menstrual cycles. According to the patients' cycle day and urinary luteinizing hormone (LH) surges, collection of proliferative and luteal phase samples was timed. Additionally, nine fresh biopsies of endometria in the proliferative phase were processed for primary cultures. All subjects were treated without any hormones for at least 3 months before testing.

Primary cell culture, transfection, siRNA-mediated *PER1* silencing and decidualization treatments

Samples were isolated and cultured as described previously (Muter *et al.* 2015). According to the manufacturer's instructions, RNA interference experiments were carried out, generally with 10 mM siRNA and Lipofectamine RNAiMAX (Invitrogen). Twenty-four hours after transfection, primary HESCs were decidualized in DMEM/F-12 containing 2% charcoal-stripped FBS (CS-FBS, Biological Industries, HaZafon, Israel), 0.5 mM dibutyryl cAMP (db-cAMP, MCE, Princeton, USA) (a cAMP analog) and 1 μM medroxyprogesterone 17-acetate (MPA, Sigma) (a progesterone analog), and cells were collected for up to 6 days for RNA or protein extraction with medium replacement every 48 h. The sequence of the siRNA is listed in Table 1. Scramble siRNA was used as a negative control. All experiments were carried out before the fifth cell passage. The volumes of fifty cells in each group were determined by using ImageJ software.

Immortalized HESC culture and decidualization treatments

An immortalized HESC line was purchased from the American Type Culture Collection (ATCC® CRL-4003™)

Table 1 Sequences used in this study.

Gene	Forward primer	Reverse primer
siRNA sequence		
PER1	GTGGGAGTCTTCTATGGTT	/
CRISPR/Cas9 sgRNA		
PER1	GGCCACGCTGCAGTACGCAC	/
PGR	AAGACGAAAGTTACGACGGC	/
ChIP-PCR primer		
PER1-PRE-1	CTCTGTTGTATGACTTGTGACT	CAAAGGGCTGGGATTACTG
PER1-PRE-2	GGCTCACGCCTGTAATCTTAT	ATGCCCCCGCTAGTTTT
PER1-PRE-3	TGTACAAAGTGCGCCTTA	ATAGGCTGAGAGGTCTTGG
PER1-PRE-4	TGGCCTGCTGACCAATGA	CCCACGCTTATTGGCTTAG
Overexpression vector primer		
FOXO1	GGCCACTAGTATGGATTACAAGGATGACGACGATAA GATGGCCGAGGCGCCTCAGGT	ATGGATCCTCAGCCTGACACCCAGCTAT
qPCR primer		
GAPDH	ACGGATTGGTCGTATTGGG	CGCTCCTGGAAGATGGTGAT
PER1	TCAAGTGGCAATGAGTCCAA	TGTAGGCAATGGAAGTCTG
PER2	TGAATGGATACGCGGAATTT	AGCTCCTTCCCACTGTCTG
PER3	CCTGTCTGTCTCTGTGTGTC	GCTGCTTCTCGAAGTAATGA
CRY1	TTGCTTGATGCAGATTGGAG	GGGAAGCCTCTTAGGACAGG
CRY2	GCCTGGATAAGCACTTGGAA	GTCCACACGGCGGTAGTAGA
CLOCK	AGCATGGTCCAGATTCCATC	ATGACTGCCCCACAAGCTAC
BMAL1	GCCATCTCGATTATGTTCTG	CTCATTGTCTGGTTCGTTGT
PRL	CTACATCCATAACCTCTCCTCAG	GGGCTTGCTCCTTGTCTTC
IGFBP1	AGAGTCGTAGAGAGTTTAGC	ACACTGTCTGCTGTGATAA
FOXO1	GGCAGCCAGGCATCTCAT	TGGGTGAGGCGGTTTCATAC
PGR	TGTATTTGTGCGTGTGGGTG	TACAGCCCATTCCCAGGAAG
DKK1	TTCCAACGCTATCAAGAACCTGC	TGCCGCACTCCTCGTCTCT
THBS1	CTGTGCGGGCAGAAAAGGG	TTGGACACCACGCTGAAGACC

BMAL1, aryl hydrocarbon receptor nuclear translocator like; *CLOCK*, clock circadian regulator; *CRY1*, cryptochrome circadian regulator 1; *CRY2*, cryptochrome circadian regulator 2; *DKK1*, dickkopf WNT signaling pathway inhibitor 1; *FOXO1*, forkhead box O1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *IGFBP1*, insulin-like growth factor-binding protein 1; *PER1*, period 1; *PER2*, period 2; *PER3*, period 3; *PGR*, progesterone receptor; *PRE*, progesterone response element; *PRL*, prolactin; *THBS1*, thrombospondin 1.

and cultured as described previously (Liao *et al.* 2015). To synchronize the circadian transcriptome (Reddy *et al.* 2007), immortalized HESCs were treated with 100 nM dexamethasone (Sigma-Aldrich) for 30 min, then treated with 10 nM estradiol to represent an undifferentiated stromal cell state or with 0.5 mM db-cAMP plus 1 μ M MPA to induce decidualization, respectively. The cells were cultured for up to 8 days for RNA or protein extraction with medium replacement every 48 h. All experiments were carried out in ten passage of the stromal cells.

Knockout of the PER1 and PGR genes with the CRISPR/Cas9 system

The sgRNAs target PER1 gene (NC_000017.11) and PGR gene (NC_000011.10) were designed on the website (<https://zlab.bio/guide-design-resources>). Each 20 bp target sequence was subcloned into the pL-CRISPR.EFS. GFP vector (Addgene plasmid #57818) to construct sgRNA expression vectors. The CRISPR/Cas9 target sequences are

listed in Table 1. HEK293T cells were plated in 15 cm culture dishes 1 day before transfection. When the cells reached 80% confluence, they were transfected with the lentivirus packaging plasmid psPAX2 (Addgene plasmid #12260), pMD2.G (Addgene plasmid #12259), and the sgRNA plasmids with Lipofectamine™ 3000 reagent (Invitrogen) according to the manufacturer's instructions. Seventy-two hours after transfection, the virus was concentrated by ultracentrifugation and then used to infect immortalized uterine stromal cells. Seventy-two hours after infection, these cells were trypsinized and single fluorescence-positive cell was selected for propagation. After several days of culturing, the genomic DNA in the clone derived from single fluorescence-positive cell was extracted for amplifying the sequences that crossing the splice sites with a high-fidelity enzyme (Toyobo, Osaka, Japan), and the PCR products were ligated into the pEASY-T cloning vector (Transgen, Beijing, China) for Sanger sequencing to confirm the knockout of the target genes. Clones with frameshift mutations in both alleles were chosen for the further experiments.

CHIP-PCR

Formaldehyde (1%) was used to fix HESCs in 10 cm culture dishes for 10 min at room temperature, and then the fixation was stopped with 125 mM glycine on ice for 10 min. After Dulbecco's phosphate-buffered saline (DPBS) washes, the cells were scraped and centrifuged for 5 min at 700g and 4°C. The sediment was resuspended in 1 mL of lysis buffer 1 (0.25% Triton X-100, 0.5% NP-40, 10% glycerol, 1 mM EDTA, 50 mM HEPES and 140 mM NaCl (pH 7.55)) and rotated for 10 min before centrifugation for 5 min at 1400g and 4°C. The sediment was then resuspended in 1 mL of lysis buffer 2 (1 mM EDTA, 0.5 mM EGTA, 200 mM NaCl, and 10 mM Tris-HCl (pH 8.0)) and rotated for 10 min at room temperature before centrifugation for 5 min at 1400g and 4°C. The sediment was resuspended in 600 µL of lysis buffer 3 (0.5% N-lauroylsarcosine, 0.1% sodium deoxycholate, 1 mM EDTA, 0.5 mM EGTA, 100 mM NaCl, and 10 mM Tris-HCl (pH 8.0)) and sonicated for 10 min at 4°C in a Bioruptor® Pico sonication device (Diagenode, Liège, Belgium). The buffers were supplemented with protease and phosphatase inhibitor cocktails (Roche). Later, 60 µL of 0.25% Triton X-100 were added and the mixture was centrifuged for 10 min at 20,000g and 4°C, the supernatant was then complexed overnight at 4°C with a progesterone receptor antibody (Cell Signaling Technology) bound to Protein A Dynabeads. Polyclonal anti-rabbit IgG was used as a negative control. After complexing, the samples were eluted successively at room temperature with the following buffers: low-salt buffer (1% Triton X-100, 0.5% sodium deoxycholate, 2 mM EDTA, 50 mM HEPES, and 150 mM NaCl); high-salt buffer (1% Triton X-100, 0.5% sodium deoxycholate, 2 mM EDTA, 50 mM HEPES, and 500 mM NaCl); LiCl buffer (0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, 250 mM LiCl, and 10 mM Tris-HCl (pH 8.0)); and Tris-EDTA buffer (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA). Then, the chromatin was eluted with 200 µL of elution buffer (1% SDS and 10 mM NaHCO₃). After an overnight incubation at 65°C to reverse-crosslink the proteins and the DNA, 0.2 mg/mL protease K was added for incubation at 37°C, and before DNA purification, the sample was incubated for 1 h at 55°C. The purified DNA was amplified by PCR, and the primer sequences are listed in Table 1.

FOXO1 overexpression plasmid construction

The human FOXO1 primer sequences for PCR are listed in Table 1. The coding sequence of the FOXO1 gene (NC_000013.11) was amplified by PCR from the cDNA of

immortalized HESCs, and the PCR product was inserted between the SpeI and BamHI sites of the pLVX-IRES-ZsGreen vector. The FOXO1 overexpression plasmid was extracted with a HiPure Plasmid EF Maxi Kit (Magen, Guangzhou, China). Sequencing and comparison with GenBank sequences were performed. HEK293T cells were plated in 15 cm culture dishes 1 day before transfection. When the cells reached 80% confluence, they were transfected by the lentivirus packaging plasmid psPAX2, pMD2.G, and the FOXO1 overexpression plasmid with Lipofectamine™ 3000 reagent according to the manufacturer's instructions. Seventy-two hours after transfection, the virus was concentrated by ultracentrifugation and then used to infect primary HESCs for 24 h. Primary HESCs were then transfected with siPER1 for 24 h, and then treated with cAMP plus MPA up to 4 days.

Dual-luciferase reporter assay

The assay was performed using two reporter vectors containing the firefly luciferase vector pGL3-Basic and the Renilla luciferase vector pRL-TK (Promega). Four PER1 promoter regions were respectively amplified by PCR from human genomic DNA and were subcloned in the upstream of the luciferase gene of pGL3-Basic vector. HEK293T cells were plated into 24-well plates, each plate was transiently co-transfected by these two vectors with or without a PR overexpression vector (pCMV-HA-PR) (Xin *et al.* 2018) and MPA using the Lipofectamine™ 3000 reagent. Thirty-six hours after transfection, cell lysates were harvested, and then luciferase activities were measured by the Dual-Luciferase Reporter System (Promega) according to the manufacturer's instructions. The firefly luciferase activities were normalized to the activities of Renilla luciferase.

RNA isolation and qRT-PCR

Total RNA was extracted using RNAiso Plus (TaKaRa) according to the manufacturer's protocol. Total RNA (1 µg) from each sample was treated with DNase I and reverse transcribed using the PrimeScript™ RT reagent Kit (TaKaRa). Real-time PCR assay was performed using TB Green® Premix Ex Taq™ II (TaKaRa) with the QuantStudio 5 Real-Time PCR System (Applied Biosystems). The analysis of amplification curve and melting curve were performed to determine the optimal primer concentration and specificity of amplifications. The primer sequences used are listed in Table 1. The analysis of standard curve was performed for estimating the efficiency of the amplifications and ranges of the assay. Real-time PCR

assay both with mRNA template and without cDNA template were tested as negative control. The variances of cDNA was normalized against the levels of the *Homo sapiens GAPDH* housekeeping gene. The expression levels of the samples were calculated using the $\Delta\Delta C_t$ method. All measurements were performed at least in triplicate, the average of the replication was used for statistical analysis.

Immunofluorescence staining

HESCs were fixed with absolute methanol at room temperature for 10 min. After three washes with PBS, the cells were permeabilized with 0.2% Triton X-100 in 1× PBS for 15 min and blocked with 0.5% (w/v) bovine serum albumin (BSA, Sigma-Aldrich) for 1 h at room temperature. Anti-PER1 (Abcam), FOXO1 (Cell Signaling Technology), Vimentin (Epitomics, Burlingame, USA), and cytokeratin (DAKO) primary antibodies were used, and then the cells were incubated with fluorescence (cyanine 3)-conjugated secondary antibodies to visualize the signals with DAPI-stained nuclei (1 µg/mL, Sigma-Aldrich). Full descriptions of the antibodies, including catalog numbers and concentrations, can be found in Table 2. The cells without primary antibody served as negative control.

Western blot analysis

Protein extraction and Western blot analysis were performed as described previously (Zhang *et al.* 2014). The antibodies used are listed in Table 2. The intensity of bands was determined by Image Lab software, and quantitative analysis of the grayscale value of each target protein was normalized to GAPDH. The gel calculation was based on three different replications, and the average grayscale value of the replication was used for statistical analysis. Only one representative film was shown in our manuscript.

Table 2 Information on the antibodies used in this study.

Antibody	Manufacturer	Catalog	Applications	
			WB	IF
PER1	Abcom	Ab136451	1:500	1:400
PRL	R&D	AF682	1:10	/
IGFBP1	Abcom	Ab181141	1:1000	/
FOXO1	CST	2880	1:1000	1:200
PR	CST	8757	1:500	/
Akt	CST	4691S	1:1000	/
p-Akt (Ser473)	CST	4060S	1:1000	/
GAPDH	Abmart	P30008	1:2000	/
Vimentin	Epitomics	2707-1	/	1:200
Cytokeratin	DAKO	Z0622	/	1:200

Akt, AKT serine/threonine kinase 1; FOXO1, forkhead box O1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGFBP1, insulin-like growth factor-binding protein 1; p-Akt, phospho AKT serine/threonine kinase 1; PER1, period 1; PR, progesterone receptor; PRL, prolactin.

Protein degradation assay

To determine the half-life of FOXO1 protein, PER1 knockout and control (empty vector) immortalized HESCs with 30–40% confluency were seeded in 3.5 cm culture dishes a day before induction of decidualization for 2 days followed by treatment with cycloheximide (CHX, EMD Millipore), a protein synthesis inhibitor, for 0, 1, 2 or 4 h. The same treatments were implemented with the calpain inhibitor MG101 (Selleck), the proteasome inhibitor MG132 (Selleck), and the autophagy inhibitor 3-methyladenine (3-MA, Selleck). A group treated with DMSO served as the negative control group.

Statistical analysis

These data are derived from entirely separate samples and are presented as means $\pm$ S.E.M. (standard error of the mean). *n* represents the number of independent experiments. Statistical analysis was performed using GraphPad Prism 8 Software and SPSS 17.0 Software. Analysis was performed with Student's unpaired two-tailed *t*-test, one-way ANOVA with Bonferroni's *post hoc* test or two-way repeated-measures ANOVA with Bonferroni's *post hoc* test. A level of *P* < 0.05 was taken to indicate statistical significance.

Results

Circadian gene PER1, rather than other clock genes, senses decidual signals

We measured the expression levels of seven core clock genes every 4 h for 32 h in immortalized HESCs *in vitro*. In contrast to the results of continuous decidualization for 4 days (Muter *et al.* 2015), these core clock genes did not exhibit obvious rhythmic activity in undifferentiated

culture. Furthermore, they did not significantly flatten in decidualized culture, indicating that the rhythm of core circadian genes may be unnecessary for the proliferation–differentiation transition in early decidualization. Nonetheless, we noticed that no statistically significant differences in every time point of these core clock genes between undifferentiated state and decidualized state, except for the elevated expression of *PER1* mRNA at 4, 8, 28 and 32 h (Fig. 1A), showing that *PER1* seems to be involved in the initiation of decidualization.

Using an *in vitro* decidualization system, the mRNA and protein levels of prolactin (PRL) and insulin-like growth factor-binding protein-1 (IGFBP1), two reliable decidual markers, increased steadily in decidualized immortalized HESCs for 2, 4, 6, or 8 days, accounting for the robustness of the experimental system (Supplementary Fig. 1A, B, C, D and E, see section on [supplementary data](#) given at the end of this article). We profiled the expression of *PER1* in this scheme and observed that the *PER1* transcript level was substantially augmented in decidualized cells (Fig. 1B). Western blot analysis also confirmed the dramatic elevations in *PER1* of up to three-fold over a 6-day period (Fig. 1C and D). Immunofluorescence staining showed that *PER1* protein was weakly expressed in the cytoplasm and nuclei of stromal cells; however, it was predominantly expressed in the nuclei of stromal cells after differentiation for 4 days, consistent with the patterns in decidua cells in early pregnancy *in vivo* (Fig. 1E). These data show that the upregulation of *PER1*, rather than other clock genes, is a striking feature of decidualization and may also be involved in the maintenance of decidualization.

Furthermore, we measured *PER1* expression levels in human endometrial biopsy samples from each phase of the menstrual cycle and found that *PER1* mRNA expression increased significantly in the mid-secretory phase of the cycle (Fig. 1F). Next, we compared the *PER1* expression of mid-luteal endometrial biopsies obtained from nine women with infertility diagnosed with EMS, a chronic syndrome characterized by infertility due to P₄ resistance and disrupted decidualization (Wetendorf & Demayo 2014), and ten women with normal fertility. The endometrial *PER1* mRNA expression in EMS patients was significantly lower than that in control patients (Fig. 1G), indicating that *PER1* may be related to P₄ responsiveness.

PER1 expression is regulated by progesterone as early as the onset of decidualization

To assess the impacts of P₄ and cAMP signaling on *PER1* expression on the onset of decidualization, we first

treated immortalized HESCs with db-cAMP and MPA separately or in combination for 6 h. The increase in *PER1* expression was more pronounced with MPA than with db-cAMP and combined treatment resulted in an additive effect (Fig. 2A). Next, we examined whether RU486 (a PR inhibitor) could diminish P₄-activated *PER1* expression. The *PER1* mRNA levels were significantly lower in RU486-treated cells than in control cells exposed to MPA alone or to a combination of db-cAMP and MPA (Fig. 2B and C), indicating that the expression of *PER1* mRNA is regulated mainly by P₄-PR signaling from the beginning of decidualization.

Considering that RU486 is also an antagonist of the glucocorticoid receptor, which is expressed in uterine stromal cells (Whirledge *et al.* 2015) and that glucocorticoids can increase *PER1* levels in a variety of cell types (Reddy *et al.* 2012, Cheon *et al.* 2013), an immortalized PGR-knockout (PGR-KO) HESC line was constructed with CRISPR/Cas9 technology to confirm this potential regulation. The Sanger sequencing results confirmed that 16 nucleic acids were inserted into and that 170 nucleic acids were deleted from coding sequence of one allele, while six nucleic acids were inserted into and 160 nucleic acids were deleted from the coding sequence of the other allele; these changes resulted in frameshift mutations and mutants with predicted deletions of 333 and 303 amino acids, respectively (Fig. 2D). Decidualized control (empty vector) and PGR-KO cell lines were analyzed by qRT-PCR and Western blot, and the results showed decreased mRNA expression of *PRL* and *IGFBP1* and a loss of PRA/PRB and *PER1* protein in PGR-KO cells (Fig. 2E and Supplementary Fig. 2A and B). Induction by MPA for 6 h attenuated the increase in the *PER1* mRNA level in the MPA-treated group compared to the control group (Fig. 2F).

PER1 is related to P₄ signal responsiveness

To ascertain whether *PER1* is regulated by PR and *PER1* is related with P₄-PR signaling responsiveness in decidualization, a *PER1*-knockout (*PER1*-KO) immortalized HESC line was constructed using the CRISPR/Cas9 system. The Sanger sequencing results confirmed that two nucleic acids were deleted from the coding sequence of one allele and that 40 nucleic acids were deleted from the coding sequence of the other allele, resulting in the predicted proteins being shortened to 210 and 213 amino acids, respectively (Fig. 3A). Control (empty vector) and *PER1*-KO cell lines were analyzed by Western blot analysis, and loss of the *PER1* protein

Figure 1

The expression of the circadian gene *PER1* increased immediately and remained elevated during decidualization. (A) Immortalized HESCs were first treated with dexamethasone for 30 min and then with estradiol (undifferentiated cells) or db-cAMP plus MPA (decidualized cells) for 32 h, and the transcript levels of seven core clock genes were measured at the indicated time points ($n = 3$ per independent experiment). The values are normalized to the GAPDH expression level. Analysis was performed between the undifferentiated group and the decidualized group using two-way repeated measures ANOVA with Bonferroni's *post hoc* test. $*P < 0.05$, $**P < 0.01$. (B, C and D) The mRNA and protein levels of *PER1* in immortalized HESCs after treatment with MPA plus db-cAMP for 0, 2, 4 or 6 days ($n = 4$ per independent experiment). The values are normalized to the GAPDH expression level. Analysis was performed with one-way ANOVA and Bonferroni's *post hoc* test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (E) Immunofluorescence staining of *PER1* in immortalized HESCs after treatment with MPA plus db-cAMP for 0, 2, and 4 days. Human decidual cells cultured *in vitro* during early pregnancy were used as positive control. The sample without primary antibody was served as a negative control. Scale bars, 100 μM . (F) qRT-PCR analysis of *PER1* mRNA levels in endometria from the proliferative ($n = 12$ samples), early secretory ($n = 6$ samples), mid-secretory ($n = 10$ samples), and late secretory ($n = 9$ samples) stages of the menstrual cycle. The values are normalized to the GAPDH expression level. Analysis was performed with one-way ANOVA and Bonferroni's *post hoc* test. ES, early secretory phase; MS, mid-secretory phase; LS, late secretory phase; P, proliferative phase. $**P < 0.01$. (G) Endometrial *PER1* transcript levels during the mid-secretory phase in fertile women ($n = 10$ samples) versus infertility patients with endometriosis (EMS) ($n = 9$ samples). The values are normalized to the GAPDH expression level. Analysis was performed with Student's unpaired two-tailed *t*-test. $*P < 0.05$.

was observed in *PER1*-KO cells (Fig. 3B). We found that *PGR* mRNA levels were not affected in *PER1*-KO cells after combined db-cAMP and MPA treatment (Fig. 3C), but the expression levels of the *DKK1* and *HTBS1*,

two PR downstream genes have been identified (Mazur *et al.* 2015), were significantly decreased (Fig. 3D and E). These results suggest that *PER1* may be associated with decreased P_4 -PR signaling responsiveness.

Figure 2

PER1 expression is regulated by progesterone as early as the onset of decidualization. (A) *PER1* mRNA levels in immortalized HESCs at 6 h after treatment with MPA and db-cAMP separately or in combination ($n = 3$ per independent experiment). The values are normalized to the GAPDH expression level. Analysis was performed with one-way ANOVA and Bonferroni's *post hoc* test. $**P < 0.01$, $***P < 0.001$. (B) *PER1* mRNA levels in immortalized HESCs at 6 h after treatment with MPA with or without RU486 ($n = 5$ per independent experiment). The values are normalized to the GAPDH expression level. Analysis was performed with Student's unpaired two-tailed *t*-test. $*P < 0.05$. (C) *PER1* mRNA levels in immortalized HESCs at 6 h after treatment in combination of MPA and db-cAMP with or without RU486 ($n = 3$ per independent experiment). The values are normalized to the GAPDH expression level. Analysis was performed with Student's unpaired two-tailed *t*-test. $**P < 0.01$. (D) Schematic diagram of the PGR knockout cell line DNA sequence by Sanger sequencing. (E) Control (empty vector) and PGR-knockout (PGR-KO) cell lines were analyzed by Western blotting after treatment with MPA plus db-cAMP for 4 days. (F) *PER1* mRNA levels of control and PGR-KO HESCs after treatment with MPA at 6 h ($n = 5$ per independent experiment). The values are normalized to the GAPDH expression level. Analysis was performed with Student's unpaired two-tailed *t*-test. $*P < 0.05$. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0284>.

Progesterone receptor modulates *PER1* via direct transcriptional activation

A previous study revealed *Per1* as a direct transcriptional target of PR in the mouse uterus (Rubel *et al.* 2012); we hypothesized that this regulatory mechanism also exists in the human endometrium. Using two reported progesterone

response elements (PREs), 5'-ACNNNNNGT-3' (Lambert *et al.* 2018) and 5'-TGTNCN-3' (Ballare *et al.* 2013), we found four putative PRE-binding sites located in the *PER1* promoter (Fig. 4A and B). After cells were allowed to decidualize for 24 h, chromatin immunoprecipitation (ChIP) analysis using a PR antibody showed that decidualization was associated with conspicuous binding

Figure 3

PER1 is related to P_4 signal responsiveness. (A) Schematic diagram of the *PER1*-knockout (*PER1*-KO) cell line DNA sequence by Sanger sequencing. (B) Control (empty vector) and *PER1*-KO cell lines were analyzed by Western blotting. (C, D and E) *PGR*, *DKK1* and *THBS1* mRNA levels in control and *PER1*-KO HESCs after treatment with MPA plus db-cAMP at 0, 2, 4, and 6 days ($n = 3$ per independent experiment). The values are normalized to the GAPDH expression level. Analysis was performed between the control group and the *PER1*-KO group using two-way repeated-measures ANOVA with Bonferroni's *post hoc* test. $*P < 0.05$, $***P < 0.001$. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0284>.

Figure 4

The progesterone receptor modulates PER1 via transcriptional activation. (A and B) Schematic diagram and sequence of the putative progesterone response element (PRE) on the PER1 promoter. (C) Chromatin immunoprecipitation-PCR (ChIP-PCR) analysis of immortalized HESCs after treatment with MPA plus db-cAMP for 24 h. The input:IgG:PR ratio was 1:12:12. (D) Schematic diagram of the pGL3-PER1-PRE-2 and pGL3-PER1-PRE-3 reporter constructs for the promoter assay. (E) Luciferase assays of the *cis*-activation potential of the region containing pGL3-PER1-PRE-2 and pGL3-PER1-PRE-3 in the presence of control plasmid, PR overexpression plasmid or MPA ($n = 3$ per independent experiment). Analysis was performed from intra-group comparison of pGL3-Basic, pGL3-PER1-PRE-2 and pGL3-PER1-PRE-3 with one-way ANOVA and Bonferroni's *post hoc* test. $*P < 0.05$. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0284>.

of PR at the locus PER1-PRE-2 (bp -1780 to -1644) and PER1-PRE-3 (bp -500 to -350) (Fig. 4C). These two sequences were inserted into luciferase reporter vectors (Fig. 4D), and we found that the activity of the reporter with the PER1-PRE-3 insert was significantly higher than that of both the PER1-PRE-2 reporter and the control reporter; in addition, the reporter with PER1-PRE-3 was actively induced by P₄-PR signaling (Fig. 4E). These results show that the expression of PER1 is potentially regulated by direct binding of PR to the PER1-PRE-3 sequence.

PER1 deficiency leads to impaired HESC decidualization via decreased FOXO1 protein levels

To investigate the function of PER1 in decidualization, siRNA-mediated knockdown was performed on primary HESCs. Successful establishment of primary stromal cell cultures was confirmed by positive staining for vimentin and negative staining for cytokeratin (Supplementary Fig. 2C). The qRT-PCR and Western blot analysis results confirmed that PER1 mRNA and protein levels were significantly lower (Fig. 5A and B) and that decidualized cells were also significantly smaller in the knockdown group than in the control group (Fig. 5C and D). These changes were accompanied by severely compromised induction of IGFBP1 and PRL at the mRNA levels (Fig. 5E and F).

Since critical decidual markers such as *Foxo1*, *Wnt4* and *Stat3* have been reported to be regulated transcriptionally by Per1 in the mouse liver (Koike *et al.* 2012), we assessed the mRNA and protein levels of these molecules after PER1 knockdown. Intriguingly, PER1

knockdown in primary HESCs exerted no significant impact on the transcripts of these genes (data not shown) and inhibited only FOXO1 protein expression (Fig. 6A, B and C). Furthermore, the immunofluorescence staining results showed that FOXO1 was strongly expressed in the nuclei and cytoplasm of decidual cells in the control group after 6 days of decidualization, but distinctly decreased in both the nuclei and cytoplasm of cells in the siPER1 group (Fig. 6D). The declines in the levels of the FOXO1, PRL, and IGFBP1 proteins were also observed in PER1-KO cells (Supplementary Fig. 2D, E, F and G), consistent with the results in knockdown cells. In primary HESCs with PER1 knockdown and FOXO1 overexpression, the aberrant decidualization was attenuated, and IGFBP1 expression levels were rescued (Fig. 6E, F and G), manifesting that PER1 promotes decidualization by regulating FOXO1.

PER1 regulates FOXO1 protein stability through the calpain pathway

To demonstrate whether the existence of another regulatory mechanism underlying the decreases of FOXO1 protein levels in the absence of PER1, as shown in Fig. 7A, B and C, the protein levels of Akt and phosphorylated Akt, the upstream regulator of FOXO1 was estimated and in siCON and siPER1 primary decidualizing cells. The comparable level of p-AKT in both groups indicated that the mRNA or protein stability of FOXO1 could be regulated by PER1 deficiency.

The half-life of FOXO1 protein was measured after inhibition of total protein synthesis. When control and

Figure 5

PER1 is indispensable for HESC decidualization. (A) *PER1* mRNA levels in primary HESCs after transfection with siRNAs for 24 h followed by treatment with MPA plus db-cAMP at 0, 2 and 4 days ($n = 3$ per independent experiment). The values are normalized to the GAPDH expression level. Analysis was performed between the control group (siCON) and the siPER1 group using two-way repeated-measures ANOVA with Bonferroni's *post hoc* test. $*P < 0.05$. (B) PER1 protein levels in primary HESCs after transfection with siRNAs for 24 h. (C) Morphology of primary HESCs after transfection with siRNAs for 24 h followed by treatment with MPA plus db-cAMP at 2, 4 and 6 days. The size of a single decidual cell is outlined with a white dotted line. Scale bars, 100 μm . (D) The cell size of primary HESCs after transfection with siRNAs for 24 h followed by treatment with MPA plus db-cAMP for 6 days ($n = 50$ cells). Analysis was performed with Student's unpaired two-tailed *t*-test. $*P < 0.05$. (E and F) *PRL* and *IGFBP1* mRNA levels in primary HESCs after transfection with siRNAs for 24 h followed by treatment with MPA plus db-cAMP at 0, 2 and 4 days ($n = 3$ per independent experiment). The values are normalized to the GAPDH expression level. Analysis was performed between the control group and the siPER1 group using two-way repeated measures ANOVA with Bonferroni's *post hoc* test. $*P < 0.05$, $**P < 0.01$. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0284>.

PER1-KO HESCs were treated with CHX for 0, 1, 2, or 4 h after decidualization for 2 days, the most remarkable observation was that the half-life of FOXO1 protein was 1 h shorter in PER1-KO cells than in control cells (Fig. 7D), suggesting that PER1 knockout affects FOXO1 protein stability. Treatment of cells with the calpain inhibitor MG101, the proteasome inhibitor MG132, the autophagy inhibitor 3-MA, or all three compounds revealed that MG101 could efficiently attenuate the rapid degradation of FOXO1 protein in PER1-KO cells, while the combination of the three compounds had no synergistic effect (Fig. 7E). These findings indicate that PER1 regulates FOXO1 protein stability mainly through the calpain-mediated degradation pathway.

Discussion

Female reproduction in humans is associated with a monthly cyclic process. With follicle growth and ovulation each month, the dynamic secretion of estradiol and P_4 plays a dominant role in orchestrating changes in uterine epithelial cells and stromal cells that prepare the endometrium for a successful pregnancy. It was first reported in 2002 that circadian genes are expressed in the mouse uterus (Johnson *et al.* 2002). P_4 treatment can

induce high expression of *Per1* in the epithelium and stroma of the rat uterus (He *et al.* 2007). In addition, the clock protein PER1 is upregulated along with decidual transformation of HESCs (Muter *et al.* 2015). Likewise, our results proved that the expression of *PER1* increased immediately and remained elevated during decidualization *in vivo* and *in vitro* due to transcriptional activation by PR, suggesting that PER1 is the major response factor for P_4 -PR signaling. *Per1*-mutant female mice show normal numbers of implantation sites but have reduced litter sizes (Pilorz & Steinlechner 2008), confirming that *Per1* deficiency may lead to unsustainable pregnancy. Decidualization provides an immune-privileged microenvironment that enables embryo implantation as well as abundant nutrients that facilitate early embryonic development, thereby maintaining gestation (Zhang *et al.* 2013), the compromised decidualization might thus be related to postimplantation loss resulting from the *Per1* mutation. In agreement with this idea, we found that *PER1* levels were decreased in the eutopic endometrium of patients with EMS, which is relevant to the finding that P_4 resistance causes decreased fecundity.

In addition to the progesterone signaling pathway, which is activated by P_4 produced by the corpus luteum, the cAMP signal transduction cascade is activated by local molecules of the endometrium in the initiation

Figure 6

PER1 deficiency leads to decreased FOXO1 protein levels. (A, B and C) FOXO1 mRNA and protein levels in primary HESCs after transfection with siRNAs for 24 h followed by treatment with MPA plus db-cAMP at 0, 2, 4 and 6 days ($n=3$ per independent experiment). The values are normalized to the GAPDH expression level. Analysis was performed between the control group (siCON) and the siPER1 group using two-way repeated-measures ANOVA with Bonferroni's post hoc test. $*P<0.05$. (D) FOXO1 immunofluorescence staining of primary HESCs after transfection using siRNAs for 24 h followed by treatment with MPA plus db-cAMP for 6 days. The primary HESC after treatment with MPA plus db-cAMP for 6 days without incubation by primary antibody was served as a negative control. Scale bars, 100 μ M. (E, F and G) Primary HESCs were infected with a FOXO1-overexpressing virus (oeFOXO1) for 24 h, and then transfected with siPER1 for 24 h and next treated with MPA plus db-cAMP for 4 days ($n=3$ per independent experiment). The FOXO1 and IGFBP1 protein levels were detected by Western blotting. The values are normalized to the GAPDH expression level. Analysis was performed with one-way ANOVA and Bonferroni's post hoc test. $***P<0.001$.

of decidualization, sensitizing endometrial cells to the actions of progesterone (Gellersen & Brosens 2003). P₄ is only a weak inducer of the decidual phenotype unless combined with cAMP, but it plays a critical role in maintaining the decidual phenotype (de Ziegler *et al.* 1998). The mRNA levels of *PER1*, a direct target gene of PR, increased rapidly when decidualization was induced *in vitro* for 4 h, suggesting the possibility that P₄ signaling is also involved in the initiation of decidualization. P₄ has long been known to exhibit circadian secretion, similar to other hormones (Kottler *et al.* 1989, Fujimoto *et al.* 1990); yet, the expression of its direct target gene *PER1* is arrhythmic, suggesting that P₄ is probably not a zeitgeber.

In mammals, the integrated endocrine system controls and adjusts physiological processes by secreting hormones. Previous findings have suggested that the endocrine system could serve as a major clock output (Lamia *et al.* 2011), and some rhythmic hormones can also activate the circadian system to regulate the expression of genes critical for physiological mechanisms (McNamara *et al.* 2001).

We observed sustained activation of the clock gene *PER1* in response to P₄ signaling during the decidualization of HESCs. In fact, upregulation of *PER1* is a generic feature in many tissues in response to certain types of hormonal stress (Mansuy *et al.* 2009, Mendoza *et al.* 2011, Smarr *et al.* 2013, Bohacek *et al.* 2015). This expression pattern indicates that *PER1* might be the most sensitive and susceptible effector in the clock system. Previous research has shown that both transcriptional and posttranscriptional regulation underlie the activity of clock system components, including *PER1*, and that posttranscriptional mechanisms account for more than half of the regulation (Koike *et al.* 2012), suggesting that transcriptional regulation is not the main mode of operation of the clock system. Our observation that *PER1* deficiency impairs the decidual transformation of the human endometrium through posttranslational regulation of FOXO1, a terminal differentiation-related gene that is evolutionarily conserved in eutherian animals and has acquired the ability to respond to decidualogenic signals (Park *et al.* 2016), strongly reinforces the idea that a

Figure 7

PER1 regulation of FOXO1 protein stability through the calpain pathway. (A, B and C) Akt and phosphor-Akt (Ser473) protein levels in primary HESCs after transfection with siRNAs for 24 h followed by treatment with MPA plus db-cAMP at 0, 2, 4 and 6 days (*n* = 3 per independent experiment). The values are normalized to the GAPDH expression level. Analysis was performed between the control group (siCON) and the siPER1 group using two-way repeated-measures ANOVA with Bonferroni's *post hoc* test. No statistically significant differences were detected. (D) After treatment with MPA plus db-cAMP for 2 days, immortalized HESCs were treated with CHX for 0, 1, 2 and 4 h, and the FOXO1 levels in the control (empty vector) and PER1-KO cells were detected by Western blotting (*n* = 3 per independent experiment). The values are normalized to the GAPDH expression level. Analysis was performed between the control group and the PER1-KO group using two-way repeated-measures ANOVA with Bonferroni's *post hoc* test. **P* < 0.05. (E) After treatment with MPA plus db-cAMP for 2 days, immortalized HESCs were treated with CHX for 2 h along with DMSO, MG101, MG132 or 3-MA. The FOXO1 levels in the control and PER1-KO cells were detected by Western blotting (*n* = 3 per independent experiment). The values are normalized to the GAPDH expression level. Analysis was performed with one-way ANOVA and Bonferroni's *post hoc* test. ***P* < 0.01. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0284>.

posttranslational mechanism is involved in the circadian gene regulatory network.

The circadian rhythm is a precisely tuned process. In the human uterus, downregulation of *PER2* in the endometrium marks the transition from oscillatory to nonoscillatory stroma, synchronizing the preimplantation embryo and surrounding decidual cells for fetal-maternal interactions (Muter *et al.* 2015). This mechanism provides a likely explanation for the abolished rhythms in differentiating HESCs. However, cell differentiation results in loss of rhythm in the clock systems of the testis and thymus, which are composed primarily of differentiating cells and exhibit constant rather than cyclic expression of circadian clock genes (Alvarez *et al.* 2003, Morse *et al.* 2003). *PER2* is necessary to maintain the undifferentiated state of CD34 (+) cells, while decreased *PER2* and loss of rhythm are associated with diabetes-induced propensity toward myeloid differentiation (Bhatwadekar *et al.* 2015). *Per3* has a prominent rhythm in mouse adipocyte precursor cells and restrains adipogenesis, a process of cell differentiation, by directly regulating *Klf15* expression

(Aggarwal *et al.* 2017). Similarly, endometrial stromal decidualization is also a cell differentiation process in which fusiform stromal cells in a proliferative state differentiate into cobblestone-shaped specialist decidual cells to implement embryo selection and promote embryo implantation; this process represents another promising yet disputed explanation of how the abolition of the *PER1* rhythm is related to cell differentiation.

In conclusion, our study introduces a mechanism of clock reaction to P_4 signaling that allows endometrial stromal cells to transform into decidual cells. We show that a phenomenon associated with P_4 -PR signaling triggers the uterine stromal cell proliferation-differentiation transition apart from maintaining this process. Our findings are likely relevant to compromised decidual responses that predispose women to reproductive failure.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-19-0284>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Y Zhang, Y Z Zhang, S Kong and H Wang conceived and designed the research; Y Zhang, N Meng, Y Jiang, N Yang, K Wu and J Wu performed the research; Y Zhang analyzed the results; and Y Zhang, H. Bao, Y Z Zhang, and S Kong wrote the manuscript. All authors reviewed the manuscript.

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