Melatonin-MT1 signal is essential for endometrial decidualization

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Abstract

Deficient decidualization of endometrial stromal cells (ESCs) can cause adverse pregnancy outcomes including miscarriage, intrauterine growth restriction, and pre-eclampsia. Decidualization is regulated by multiple factors such as hormones and circadian genes. Melatonin, a circadian-controlled hormone, is reported to be important for various reproductive processes, including oocyte maturation and placenta development. Its receptor, MT1, is considered to be related to intrauterine growth restriction and pre-eclampsia. However, the role of melatonin-MT1 signal in decidualization remains unknown. Here, we reported that decidual stromal cells from miscarriages displayed deficient decidualization with decreased MT1 expression. The expression level of MT1 is gradually increased with the process of decidualization induction in vitro. MT1 knockdown suppressed the decidualization level, while the overexpression of MT1 promoted the decidualization process. Moreover, changing MT1 level could regulate the expression of decidualization-related transcription factor FOXO1. Melatonin promoted decidualization and reversed the decidualization deficiency due to MT1 knockdown. Using in vitro and in vivo experiments, we further identified that lipopolysaccharide (LPS) could induce inflammation and decidualization resistance with downregulated MT1 expression, and melatonin could reverse the inflammation and decidualization resistance induced by LPS. These results suggested that the melatonin-MT1 signal might be essential for decidualization and might provide a novel therapeutic target for decidualization deficiency-associated pregnancy complications.

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Introduction

Human endometrial stromal cells (hESCs) undergo cyclic changes during each menstrual cycle including proliferation and differentiation (Brosens et al. 2002, Gellersen & Brosens 2003, Tamura et al. 2018). For better adaption to embryo implantation, the hESCs go through extensive differentiation into large, round, multi-nucleated decidual stromal cells (DSCs), a process termed as decidualization, to ensure the establishment and maintenance of a successful pregnancy (Matsumoto et al. 2009, Tamura et al. 2018). Deficient decidualization is the main cause of adverse pregnancy outcomes such as miscarriage, pre-eclampsia, and premature labor. During decidualization, the expression of IGF-binding protein 1 (IGFBP1) is significantly upregulated and used as a classical marker of decidualization level (Al-Sabbagh et al. 2011, Tamura et al. 2018). Forkhead box O 1 (FOXO1) is one part of the core regulatory transcription factor complex that establishes the cell type identity of DSCs (Kajihara et al. 2006, Park et al. 2016). Knockdown of FOXO1 inhibited progesterone-induced IGFBP1 expression and decidualization (Takano et al. 2007). FOXO1 is regarded to be a critical transcription factor in decidualization regulation. Decidualization is regulated by many physiological and pathological factors such as hormones, circadian rhythm, inflammation, and oxidative stress (Al-Sabbagh et al. 2011, Rubel et al. 2012, Zhang et al. 2019). Increased expression of pro-inflammatory cytokines ( interleukin (IL)1B, IL6, and tumor necrosis factor α (TNF)) under the exposure of lipopolysaccharide (LPS) can restrict decidualization induction (Hu et al. 2007, Lv et al. 2015, Bonney 2016). Despite plenty of molecules that have been reported to...
be involved in decidualization regulation, the potential mechanism remains largely unclear.

Melatonin (N-acetyl-5-methoxytryptamine) is a neuroendocrine hormone mainly secreted by the pineal gland and also synthesized in the ovary and placenta. It regulates not only circadian rhythm but also multiple physiological and pathological processes such as inflammation, oxidative stress, apoptosis, and metabolism (Peschke et al. 2015, Sagrillo-Fagundes et al. 2018). Recently, its roles in reproduction attract more attention. Melatonin regulates steroid hormone secretion and protects sperm viability in male reproduction (Henkel et al. 2001). Moreover, increasing studies suggest that melatonin plays critical role in female reproduction. Melatonin with high concentration is observed in follicular fluid and contributes to oocyte maturation (Tamura et al. 2012). In the process of in vitro fertilization-embryo transfer (IVF-ET), melatonin can improve oocyte quality and subsequent implantation rates (Dair et al. 2008, Tamura et al. 2014). Downregulated melatonin concentration and disrupted melatonin rhythm are observed in pregnant women with pre-eclampsia, which suggests melatonin might be involved in the pathogenesis of pre-eclampsia (Tamura et al. 2008). In addition, melatonin regulates uterine smooth muscle activity and further affects uterine contraction and labor (Olcese et al. 2013). Therefore, defective melatonin may be related to adverse pregnancy outcomes such as miscarriages, pre-eclampsia, and premature labor. Increasing evidences suggest that disrupted decidualization is a crucial cause for adverse pregnancy outcomes (Saben et al. 2016, Mei et al. 2019). It is thus important to investigate whether melatonin is involved in decidualization, which may provide a therapeutic target for decidualization deficiency-associated pregnancy complications.

MT1 and MT2 (also known as MTNR1A and MTNR1B), members of the 7-transmembrane G protein-coupled receptor family, mediate the functions of melatonin (von Gall et al. 2002). These receptors have been identified in the brain (hippocampus, cerebellum, and suprachiasmatic nucleus) and reproductive organs (ovary, uterus, and breast) (Woo et al. 2001, Sanchez-Barcelo et al. 2005, Tamura et al. 2008). Previous studies have indicated that MT1 but not MT2 was involved in circumcadian rhythm regulation (Comai et al. 2019). The expression of these receptors can be regulated by hormones such as melatonin and human choriogonadotrophin. The level of MT1 but not MT2 is rapidly upregulated by mouse choriogonadotrophin in mouse granulosa cells (He et al. 2016). Similarly, the hormone level of the uterine microenvironment influences the expression of melatonin receptors, whose level is distinct in different estrous cycle of rat (Zhao et al. 2002). In addition, inflammatory balance in the uterine microenvironment is crucial for the establishment and maintenance of pregnancy. It has been reported that decreased melatonin level in blood upregulates pro-inflammatory cytokines such as IL1B, IL6, and TNF (Sagrillo-Fagundes et al. 2018, Yang et al. 2018a). Whether the alteration of inflammatory balance affects the level of melatonin receptors is unknown, and whether melatonin receptors under proinflammatory status directly regulate the process of decidualization is also unclear.

In this study, we compared MT1 expression and decidualization level in DSCs from normal pregnancies and miscarriages to propose the relationship between MT1 and decidualization. Applying si-RNA transfection and plasmid overexpression, we investigated whether MT1 regulated decidualization. We further used the LPS-induced decidualization resistance model to suggest that the inflammatory response induced by LPS might be a cause of downregulated MT1 expression and clarify the preventive effect of melatonin on decidualization deficiency induced by LPS in vivo.

Materials and methods

Human samples

Human endometrial tissues were collected from women (n = 30) with regular menstrual cycles (25–35 days) without intrauterine abnormalities and who had not undergone exogenous steroidal hormones therapy for 3 months preceding biopsy collection (Table 1). First-trimester (gestational age: 6–12 weeks) human decidual tissues were obtained from clinically normal pregnancies (22–40 years old, terminated 6–12 weeks) human decidual tissues were obtained from clinically normal pregnancies (22–40 years old, terminated for non-medical reasons, n = 9) and miscarriages (22–40 years old, diagnosed as unexplained abortion excluding genetic, infection, endocrine and other factors. n = 9) (Table 1). All participants provided written informed consent. All performances were approved by the Human Research Ethics Committee of the Obstetrics and Gynecology Hospital of Fudan University.

Cell culture and treatment

hESCs were isolated from endometrial tissues as described (Zhao et al. 2019). Briefly, hESCs were collected from endometrial tissues digested by collagenase IV (C5138, Sigma) and cultured in complete medium (Dulbecco's modified Eagle's medium/F-12 (DMEM/F12, Logan Utah, USA) supplemented with 10% charcoal-stripped fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin (B54072, Sangon Biotech, Shanghai, China)) in a 37°C humidified incubator containing 5% CO2. For decidualization experiments (Lv et al. 2019), hESCs were treated with 1 μM MPA and 0.5 mM db-cAMP (T1418, Topscience, Shanghai, China) in complete medium for indicated time or 48 h. For siRNA (siRNA) or plasmid transfection tests, the cells were transfected with MT1 nonspecific control siRNA (Blank Control), MT1 specific siRNA (si-MT1: CGTGGCCGATAGGTTAAA) or MT1 plasmid (Genechem, Shanghai, China) for 20 h using transfection reagent (L3000-015, Invitrogen) according to the manufacturer's instructions. For melatonin test, the cells


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were treated with melatonin (HY-B0075, MedChemExpress) at different concentrations for 24 h to collect cells for detecting the mRNA levels or for 48 h to collect cells for detecting the protein levels. For LPS treatment (Zhao et al. 2019), the cells were treated with 200 ng/mL LPS (L2630, Sigma) for 24 h to collect cells for detecting the mRNA levels or for 48 h to collect cells for detecting the protein levels. For LPS and melatonin treatment, the cells were treated with 10 nM melatonin for 4 h before LPS treatment.

### Immunofluorescence
Paraffin sections of decidual tissues were washed three times with tris-buffered saline (TBS) (10 min each) after dewaxing and antigen retrieval and then incubated with 10% donkey serum for 2 h. Sections were incubated with primary antibodies (mouse anti-MT1(sc-390328, Santa Cruz); rabbit anti-vimentin (ab92547, Abcam)) overnight at 4°C, and then incubated with secondary antibodies (Alexa Fluor® 594 Donkey Anti-Mouse (715-585-150, Jackson); Alexa Fluor® 488 Donkey Anti-Rabbit (711-545-152, Jackson)) for 2 h at room temperature after washing three times with TBS. Subsequently, sections were stained with 4',6-DAPI for 7 min and washed three times with TBS. After sealing with mounting medium (AQUA-MOUNT, Lerner Laboratories, Kalamazoo, USA), images were captured using a fluorescence microscope. Mean gray value was measured using ImageJ software. Relative mean gray value = mean gray value of cells from miscarriages/the mean value of mean gray value of cells from normal pregnancies.

### Western blot
Total proteins from cells or homogenized tissues dissolved in radioimmunoprecipitation assay lysis buffer (P0013B, Beyotime, Shanghai, China) containing protease inhibitor (04806845001, Roche) and determined the protein concentration using bicinchoninic acid protein assay kit (P0010, Beyotime). Protein of 20 μg of each sample were separated by 10% sodium dodecyl sulfate polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane (ipvh00010, Millipore). After blocking with 5% non-fat dry milk in TBS with 0.1% Tween 20 (TBST) for 2 h at room temperature, the membrane was incubated with primary antibodies (anti-MT1(sc-390328, Santa Cruz); anti-β-TUBULIN (TUBB)(ab179513, Abcam); anti-IGFBP1 (ab180948, Abcam); anti-β-ACTIN (ACTB) (ab179467, Abcam)) overnight at 4°C. The membrane was washed three times with TBST and then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. At last, the membrane was exposed with an enhanced chemiluminescence (ECL) imager (Merck Millipore) using ECL kit (BF06053, Biodragon). ACTB and TUBB were used as internal standards. The band intensities were quantified by densitometry using the ImageJ software.

### Quantitative real-time PCR (QPCR)
Total RNA was extracted from cells using TRizol reagent (9109, Takara) according to the manufacturer's protocol. cDNA was obtained by RT according to the manufacturer's protocol and then amplified with SYBR Green PCR Master Mix (Takara) on ABI PRISM 7900 Sequence Detection System (Applied Biosystems). ACTB and GAPDH (or Actb and Gapdh) were internal control genes to normalize relative mRNA expression by 2−ΔΔCT method. ΔCt= Ct genes – mean of Ct internal control genes. The primers were as follows: IL1β: forward 5'-AGCTACAAGATCTCC CGACAC3' and reverse 5'-CGTITACCCATGTTGTCGAAGGA3'; IL6: forward 5'-ACTACCTCTTCAAGACAAGGATTG3' and reverse 5'-CCATCTTGGAGGATCTAGGTTG3'; TNF: forward 5'-CAGGGACCTCTCTTCAAT3' and reverse 5'-CTCAACATGGGCTGCTCAG3'; Mt1: forward 5'-TGCAGCGAGCTGCTCAATG3' and reverse 5'-GGTGATGGGAGGATACGA3'; Wnt4: forward 5'-GCAACGTTCCTGAACTCCTT3' and reverse 5'-AATGGTGCTGCTCTGCTGT3'. The primer of GAPDH (B662104-0001), ACTB (B662102-0001), Gapdh (B662304-0001) and Actb (B662302-0001) was purchased from Sangon Biotech.

### Enzyme-linked immunosorbent assay (ELISA)
The culture supernatant of hESCs was collected and centrifuged at 1000 g for 10 min to get supernatant for ELISA. The levels of inflammatory cytokines (IL1B, IL6, TNF) in the supernatant were measured using an ELISA kit according to the
manufacturer’s instructions. ELISA kits of IL6 (BDEL-0022) and TNF (BDEL-0049) were purchased from Biodragon, and IL1B (CSB-E08053h) was purchased from Cusabio. Fluorescence value and concentration of standard samples were fitted by polynomial. The concentrations of inflammatory cytokines were calculated based on the polynomial function.

Mouse model

All mice (8–10 weeks old) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. Mice were housed in a room of 22–25°C, 40–60% relative humidity, 12 h light:12 h darkness cycles and fed with food and water ad libitum. All mice experiments were approved by Institutional Animal Care and Use Committee at Fudan University. The female mice were mated with male mice with vasoligation at 19:00. At 7:00 the next morning, a vaginal plug was detected and referred to as day 0.5 of pseudopregnancy (PE0.5). For the control group, the mice were injected intraperitoneally with physiological saline at PE2.5–6.5 (once a day). For the LPS group, the mice were administrated with 0.25 mg/kg LPS at PE3.5 and physiological saline at PE2.5–6.5 (once a day). For the LPS and melatonin group, the mice were administrated with 0.25 mg/kg LPS at PE3.5 and 5 mg/kg melatonin at PE2.5–6.5 (once a day). For artificial decidualization (Yang et al. 2018b), the dorsum of female mice was shaved and disinfected with 75% alcohol at PE3.5. A 0.5 cm long dorsal incision was carefully made around the position of right uterine horn. A sterile gauze was put on the mouse dorsum and made a slit to expose the dorsal incision. Right uterine horn was injected with 25 μL sesame oil to induce decidualization and the left uterine horn served as control. All mice were sacrificed at PE7.5 to analyze the decidualization level.

Statistical analysis

All data were acquired from at least three independent replicates. Statistical analysis was performed using GraphPad Prism version 7. All data were tested for normality using Shapiro–Wilk test before statistical analysis. For parametric data, they were analyzed with a Student's t-test to compare the difference between two groups. For nonparametric data, they were analyzed with a Mann–Whitney test to compare the difference between two groups. All data showed mean ± S.E.M. P < 0.05 was considered as statistically significant difference.

Results

**MT1 downregulation was associated with deficient decidualization in human miscarriage**

To identify whether there was a correlation between MT1 and pregnancy outcomes, we analyzed the expression of MT1 in DSCs from normal pregnancies and miscarriages. As shown in Fig. 1A, a lower level of MT1 was observed in DSCs from miscarriages compared with those in normal pregnancies. Deficient decidualization is one of the important reasons for miscarriage. We detected the expression level of IGFBP1, a marker of decidualization. As expected, decreased IGFBP1 was observed in DSCs from miscarriages. Interestingly, the expression level of MT1 and IGFBP1 is positively correlated with each other based on normal pregnancy or miscarriage (Fig. 1B). Furthermore, the expression level of MT1 was gradually upregulated with decidualization process under different induction times of decidualization (Fig. 1C). These results suggested that MT1 might be important for decidualization process and normal pregnancy.

**Melatonin-MT1 regulated human decidualization process in vitro**

To define whether MT1 regulated decidualization, we analyzed the level of decidualization of hESCs with different expression levels of MT1 under the exposure of MPA and db-cAMP. First, we knocked down the expression of MT1 in hESC by transfecting siRNA of MT1 and found that silencing MT1 significantly decreased the expression of IGFBP1 in hESCs under decidualization induction (Fig. 2A). Then, we forced the expression of MT1 by transfecting the overexpression plasmid of MT1 in hESCs under decidualization induction. The results in Fig. 2B showed that the overexpression of MT1 significantly upregulated the expression of IGFBP1. These results suggested that MT1 could regulate the process of decidualization. Since FOXO1 was a critical transcriptional factor of IGFBP1 expression (Takanou et al. 2007), we wondered if MT1 could regulate decidualization via FOXO1. As shown in Fig. 2C and D, knockdown of MT1 decreased the expression of FOXO1, and overexpression of MT1 promoted expression of FOXO1, suggesting that FOXO1 might be a potential downstream transcriptional factor involved in MT1-regulated decidualization. Thinking that melatonin is the ligand of MT1, we further treated the cells with melatonin to activate MT1 signal and found that treatment with 10 nM melatonin remarkably promoted decidualization process induced by MPA and db-cAMP (Fig. 3A). Moreover, melatonin could rescue decidualization deficiency caused by MT1 knockdown (Fig. 3B). These data indicated that stimulating melatonin-MT1 signal could promote the human decidualization process in vitro.

**LPS suppressed MT1 expression and melatonin reversed the effect of LPS on inflammation and decidualization in hESCs**

The expression of MT1 was decreased in DSCs from miscarriages and MT1 played a critical role in the decidualization of hESCs (Figs 2 and 3), supposing deficient decidualization caused by decreased MT1 expression could be an important reason for...
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We then explored the potential cause for downregulating MT1 expression. It is well-known that an excessive pro-inflammatory environment is one main cause of miscarriage. To investigate whether excess inflammation altered the expression of MT1, we stimulated hESCs with LPS to induce a pro-inflammatory status and found that LPS decreased the expression of MT1 and IGFBP1 (indicated decidualization level) (Fig. 4A). Moreover, pretreatment with melatonin restored the decreased expression of IGFBP1 induced by LPS, suggesting that activating MT1 could reverse LPS-induced resistance of decidualization process (Fig. 4B). In addition, we observed that melatonin could alleviate LPS-induced production of pro-inflammatory cytokines (IL-1β, IL-6, TNFα) in hESCs (Fig. 4C, D, E, F, G and H), indicating that MT1 signaling could interrupt the bad feedback loop between inflammation and decidualization.

Melatonin relieved LPS-induced decidualization resistance and inflammation in mice

To further investigate the preventive effect of melatonin on decidualization resistance in the pro-inflammatory status, we established the in vivo decidualization model with control, LPS alone or combined LPS with melatonin treatment, respectively. Consistent with in vitro experiment, LPS decreased the expression of Mt1 and increased the expression of pro-inflammatory cytokines (Il1b, Il6, Tnf) in the mouse decidualization model. Administration with melatonin reversed these changes induced by LPS in decidual tissues (Fig. 5A and B). The decidualization level in mice uterus was also observed by uterine morphology and expression of decidual markers (Diptp, Wnt4, and Bmp2) in decidual tissues. The results in Fig. 5C, D and E demonstrated that LPS inhibited the decidualization level and melatonin

Figure 1 MT1 downregulation was associated with deficient decidualization in human miscarriage. (A) (left) Immunofluorescence for MT1 and vimentin (VIM) staining in decidual tissues from normal pregnancies and miscarriages (n = 6). (right) The relative mean gray value of MT1 in VIM+ hESCs from normal pregnancies and miscarriages (n = 3). (B) The expression of MT1 and IGFBP1 in DSCs from normal pregnancies and miscarriages were analyzed by Western blot (n = 3). (C) The expression of MT1 and IGFBP1 in hESCs under decidualization induction by MPA and db-cAMP for 12, 24, 48, and 72 h (n = 3). NP, normal pregnancies; Mis, miscarriages; MC, MPA and db-cAMP. Black dots represented raw data. Data represented mean ± s.e.m. a, b, and c represented P < 0.05, P < 0.01, and P < 0.001, respectively.
could reverse the decidualization resistance induced by LPS. These data demonstrated that the decreased 
*Mt1* expression under the condition of inflammation might be the main cause of decidualization resistance induced by LPS, and melatonin could be a potential therapeutic drug for deficient decidualization induced by inflammation.

**Discussion**

Circadian rhythm is mainly controlled by the suprachiasmatic nucleus of the hypothalamus, a core pacemaker, in mammals (Drouyer et al. 2007, Takano et al. 2007). Its regulation relies on a transcriptional–translational loop consisted of clock genes. Melatonin, a circadian-controlled humoral factor, is also an important regulator of circadian rhythm. Melatonin mainly works through MT1 and MT2 and also plays role in antioxidation, proliferation, and anti-apoptosis (Tamura et al. 2008). Suppression of melatonin secretion can alter the expression of *MT1* and clock genes (Pfeffer et al. 2012). Previous studies have shown that the knockdown of MT1 in mouse granulosa cells induced apoptosis and suppressed proliferation, and the antioxidant role of melatonin in the ovary was mediated by MT1 (Barberino et al. 2017, Huang et al. 2019). Although it is well-known that melatonin is important for the function of the ovary and placenta, the role of melatonin and MT1 in hESCs is largely unclear. In the present study, we observed a decreased expression of MT1 in DSCs from miscarriages, accompanied by deficient decidualization. Using siRNA or plasmid interference strategy, we demonstrated that MT1 might be important in the regulation of decidualization. Melatonin could promote decidualization and reverse deficient decidualization due to the knockdown of MT1. Furthermore, we found that LPS-induced inflammation might be a cause of decreased MT1 expression and decidualization deficiency. Melatonin could reverse decidualization resistance induced by LPS.

Pregnancy is a complex physiological process, mainly involving three critical uterine events, like decidualization, vascular remodeling, and immune tolerance. Deficient decidualization could cause the failure of vascular remodeling and the imbalance of immune tolerance, leading to adverse pregnancy outcomes (Sang et al. 2020). Several researches have reported that the knockdown of circadian genes (*Per1*,

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**Figure 2** Alteration of *MT1* expression regulated decidualization process. (A) Immunoblots showing the expression of *MT1* and *IGFBP1* in hESCs with or without *MT1* knockdown under decidualization induction by MPA and db-cAMP (*n* = 3). (B) The expression of *MT1* and *IGFBP1* in hESCs with or without *MT1* overexpression under decidualization induction by MPA and db-cAMP (*n* = 3). (C) The expression of *FOXO1* and *IGFBP1* in hESCs with or without *MT1* knockdown under decidualization induction by MPA and db-cAMP (*n* = 3). (D) The expression of *FOXO1* and *IGFBP1* in hESCs with or without *MT1* overexpression under decidualization induction by MPA and db-cAMP (*n* = 3). Black dots represented raw data. Data represented mean ± s.e.m. a, b, and c represented *P* < 0.05, *P* < 0.01, and *P* < 0.001, respectively.

**Figure 3** Melatonin attenuated the decidualization deficiency due to *MT1* knockdown in hESC. (A) The expression of *MT1* and *IGFBP1* in hESCs dealt with different concentrations of melatonin under decidualization induction by MPA and db-cAMP (*n* = 3). (B) Melatonin rescued the decidualization deficiency due to *MT1* knockdown in hESC. Mel represented melatonin (*n* = 3). Black dots represented raw data. Data represented mean ± s.e.m. a and b represented *P* < 0.05 and *P* < 0.01, respectively.
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Figure 4 LPS suppressed MT1 expression and melatonin reversed the effect of LPS on inflammation and decidualization in hESCs. (A) Immunoblots showing the expression of MT1 and IGFBP1 in hESCs treated with different concentrations of LPS under decidualization induction by MPA and db-cAMP (n = 3). (B) Immunoblots showing the expression of MT1 and IGFBP1 in hESCs treated with 200 ng/mL LPS or 10 nM melatonin under decidualization induction by MPA and db-cAMP (n = 3). (C, D, and E) Relative mRNA levels of IL1B, IL6, and TNFα in hESCs treated with 200 ng/mL LPS and/or 10 nM melatonin (n = 6). (F, G, and H) ELISA for IL1B, IL6, and TNFα in supernatant of hESCs treated with 200 ng/mL LPS and/or 10 nM melatonin (n = 6). Black dots represented raw data. Data represented mean ± s.e.m. a, b, c, and d represented P < 0.05, P < 0.01, P < 0.001, and P < 0.0001, respectively, compared with control group without LPS and melatonin treatment; A, B, and D represented P < 0.05, P < 0.01, and P < 0.0001, respectively, compared with the group with LPS treatment. Mel, melatonin.

Bmal1 suppressed the process of decidualization (Lv et al. 2019, Zhang et al. 2019). The present study showed another circadian-controlled signal, melatonin-MT1, could also regulate the decidualization. FOXO1, Homeobox (HOX) A10, C/EBPB are important transcription factors involved in the regulation of decidualization (Mantena et al. 2006, Takano et al. 2007, Wei et al. 2009). Here, we found that FOXO1 might be involved in MT1-regulated decidualization. Whether MT1 regulated the decidualization of hESCs via other factors also needs to be determined in the future. Although MT1 and MT2 have different roles during the differentiation of villous cytrophoblast into syncytiotrophoblast (Yang et al. 2018a), the role of MT2 in decidualization needs to be further explored.

Melatonin plays critical role in the establishment and maintenance of pregnancy (Tamura et al. 2014). Previous study has shown that melatonin in the follicular fluid could protect oocytes and granulosa cells from oxidative stress and improve the quality of oocytes, which is required for a healthy ‘seed’ of pregnancy (Dair et al. 2008, Tamura et al. 2012). Endometrial decidualization and its related receptivity are important for the establishment of pregnancy during the window of embryo implantation. Hormone disorder and oxidative stress can induce functional changes of hESCs and cause alteration of endometrial receptivity (Madero et al. 2016, Munro 2019). We found here that melatonin and MT1 are essential for decidualization, which might further be involved in the regulation of endometrial receptivity. Although melatonin reversed the decidualization deficiency induced by MT1 knockdown, the regulatory mechanism was still unclear. Melatonin may enhance the activity of MT1 to induce the activation of downstream signal which increases the decidualization sensibility. Previous studies indicated that melatonin could scavenge reactive oxygen species (ROS) and reactive nitrogen species (RNS) via receptor-independent actions (Galano et al. 2011, 2013). The increased ROS level could induce decidualization damage (Yu et al. 2019). Therefore, we suspected that melatonin may also scavenge ROS induced by MT1 knockdown to increase the decidual level.

A successful pregnancy depends on the balance of the uterine microenvironment such as hormones balance, anti-/pro-inflammatory balance, and immune balance (Mendes et al. 2019). Inflammation caused by a variety of pathogens in the endometrium is the main cause of adverse pregnancy outcomes. LPS, the main component of the outer membrane of gram-negative bacteria, can elicit an inflammatory response (Lv et al. 2015). We demonstrated that administration of LPS decreased the expression of MT1 and induced decidualization resistance, and melatonin could reverse these effects induced by LPS treatment. This study may contribute to explore the potential therapeutic application of melatonin signal modulation for miscarriage. Low MT1 prior to pregnancy could be responsible for subsequent low levels of MT1 and IGFBP1 in miscarried tissue. This is only speculation as we are not certain if this is a cause or consequence of the pregnancy failure. To further prove it, we need to investigate the decreased decidualization level and adverse pregnancy outcomes in Mt1 conditional knockout mice that could be rescued through melatonin administration and demonstrate the decreased MT1 expression in hESCs from patients with
intrauterine inflammation, accompanied by deficient decidualization in vitro, and then track whether adverse pregnancy outcomes occur in these patients.

In conclusion, we provided evidences that MT1 was important for the decidualization process, and FOXO1 might be involved in the regulation of decidualization by the metatonin-MT1. Furthermore, LPS suppressed the expression of MT1 and decidualization process, stimulation of MT1 by melatonin could reverse the decidualization resistance induced by LPS. Our study raised a possibility that the melatonin-MT1 signal might be a potential target for diagnosis and prevention of pregnancy complications caused by inflammation-mediated decidualization deficiency.

Declaration of interest
The authors declare that there is no conflict of interest that could be prejudice the impartiality of the research reported.

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Author contribution statement
L Y C designed this project, performed experiments, analyzed data, and drafted the manuscript. F X performed experiments and analyzed data. S C W, Z X J, L L helped to collect samples and analyze data. Y D and X L S took part in discussing this project and revised the manuscript. M R D conceived this project and revised this manuscript. All authors reviewed and approved the final manuscript.

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