



MCM6 Inhibits Decidualization via Cross-Talking with ERK Pathway in Human Endometrial Stromal Cells

Yaling Jiang¹ · Yuan Xue¹ · Xinhua Yuan¹ · Shengqin Ye¹ · Mingxing Liu¹ · Yu Shi¹ · Hua Zhou¹

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Abstract

Decidualization plays an important role in the implantation of the embryo, but the molecular action implicated in this process is not completely known. Herein, we found that, compared with the proliferative endometrial tissues, the expression of minichromosome maintenance complex component 6 (MCM6) was markedly decreased in the secretory endometrial tissues. To verify the function of MCM6 in decidualization, *in vitro* decidualization model was constructed by treating human endometrial stromal cells (HESCs) with estrogen (E2) and progesterone (P4). Consistently, MCM6 level was downregulated in E2P4-treated HESCs. Administration of E2P4 accumulated HESCs in G1 cell cycle phase, leading to cell growth suppression. Ectopic expression of MCM6 promoted the transition of G1/S and restored the proliferation of HESCs that were inhibited by E2P4. MCM6 overexpression led to aberrant activation of extracellular signal-regulated kinase (ERK) and treatment with ERK agonist Ro 67–7476 restored MCM6 expression and cell proliferation inhibited by E2P4. Our data suggested that MCM6/ERK feedback loop plays a negative role in E2P4-induced decidualization and implies that MCM6 may be a promising target for meliorating uterine receptivity.

Keywords Endometrial stromal cells · Decidualization · MCM6 · ERK · Mechanism

Introduction

Infertility has become a major health problem in women with increasing prevalence [1]. Decidualization is a crucial step during embryo implantation, which is associated with the success of human pregnancy in women [2]. Accumulated studies have suggested that defective decidualization accounts for recurrent implantation failure and recurrent pregnancy loss [3]. Decidualization is controlled by a series of hormones, genetic molecules, and pathways [4, 5], but its

mechanism was unclarified. A better understanding of the biomolecular mechanisms underlying decidualization will be beneficial to improving pregnancy.

Stromal cell proliferation is crucial for endometrial decidualization, which is tightly regulated by a number of cell cycle regulators [6]. The minichromosome maintenance (MCM) proteins are well-known as DNA replication modulators, participating in many biological activities, including cell growth, apoptosis, and survival [7]. There are six MCM proteins, MCM2–7, which load onto the chromatin early at the G1 phase and trigger the origin activation when cells enter the S phase [8]. One of these members, MCM6, has been reported to limit DNA replication during the cell cycle [9]. MCM6 is crucial for cell growth, and increased expression of MCM6 has been observed in various cancer cells, including endometrial carcinoma [10, 11]. A recent study suggests that MCM2 controls stromal cell proliferation and differentiation, contributing to the differentiation of stromal cells induced by progesterone (P4) in mice [12]. However, it is still unknown whether MCM6 affects the progression of decidualization in humans.

Extracellular signal-regulated kinase (ERK) signaling plays a key role in cell survival, motility, and angiogenesis

Yaling Jiang and Yuan Xue equally contributed and both are considered the first author.

✉ Yaling Jiang
3418153001@qq.com

¹ Department of Obstetrics and Gynecology, Guangdong Provincial Key Laboratory of Major Obstetric Diseases, Guangdong Provincial Clinical Research Center for Obstetrics and Gynecology, Guangdong-Hong Kong-Macao Greater Bay Area Higher Education Joint Laboratory of Maternal-Fetal Medicine, The Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, China

[13]. Several studies have also demonstrated that ERK signaling is related to the proliferation and migration of endometrial cells [14–16]. MCM6 is reported to be an upstream regulator of ERK signaling, which can promote hepatocellular carcinoma cell migration via activation of ERK pathway [17]. Interestingly, treatment with ERK inhibitor PD98059 can inhibit mitogen-induced MCM6 expression in vascular smooth muscle cells [18]. These results imply that there exists a crosstalk regulation between MCM6 and ERK. Nevertheless, the role of MCM6/ERK cascade in decidualization is unclarified.

This study aimed to analyze the level of MCM6 in the proliferative and secretory endometrium tissues and investigate its function and mechanisms implicated in decidualization using the estrogen (E2)/progesterone (P4)-induced in vitro decidualization model.

Materials and Methods

Specimen Collection

Endometrium specimens of the proliferative ($n = 10$) and secretion endometrium ($n = 10$) were accessed by hysteroscopy from normal women from the Third Affiliated Hospital of Guangzhou Medical University. Endometrial tissues in the proliferative and secretion endometrium were confirmed by a pathologist. All women provided their written informed consent prior to the participation. The committee of the Third Affiliated Hospital of Guangzhou Medical University approved the protocol of this work [2017–177]. Human tissue samples conformed to the standards set by the Declaration of Helsinki.

Cell Culture

Human endometrial stromal cells (HESCs) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). HESCs were incubated in DMEM/F12 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 2% FBS and 1% penicillin and streptomycin. For establishing the decidualization model in vitro, E2 (10 nM) and P4 (1 μ M) were added to HESCs (2.5×10^5) cells in a 24-well plate, and incubation was conducted for 24 h, 48 h, and 72 h.

Antibodies and Reagents

Antibodies against MCM6 (ab201683), PRL (ab188229), IGFBP1 (ab228741), Cyclin D1 (ab16663), CDK2 (ab32147), Cyclin E1 (ab33911), and GAPDH (AC001) were obtained from Abcam (Cambridge, UK). Horseradish peroxidase

(HRP)-conjugated secondary antibodies and Alexa Fluor488-conjugated antibody were from Cell Signaling Technology (Beverly, CA, USA). Estrogen (E2) and progesterone (P4) were from Sigma-Aldrich (St. Louis, MO, USA).

qRT-PCR

Isolation of total RNA was conducted using HESCs and endometrium specimens in the proliferative and secretory phases. cDNA was synthesized with total RNA with an oligo (dT) 18 primer using the Transcriptor First Strand cDNA Synthesis Kit (Seyotin, Guangzhou, China). The SYBR Green PCR Real-Master Mix (Seyotin, Guangzhou, China) was utilized for PCR amplification. The reaction was undertaken under the Applied Biosystems 7300 (Applied Biosystems, Foster City, CA, USA). The PCR was run at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and elongation at 68 °C for 30 s. The $2^{-\Delta\Delta CT}$ method was used to calculate the gene expression. The real-time PCR primers were presented in the Supplemental Table 1.

DNA Transfection

The pcDNA-3.1-MCM6 plasmid was purchased from Fenghui Biotechnology (Hunan, China). HESCs (70–80% confluence) were transfected with pcDNA-3.1-MCM6 plasmid (or control vector) with Lipofectamine™ 3000 transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA). At 48 h post-transfection, HESCs were collected for further experiments.

Cell Viability Analysis

HESCs (5×10^3) transfected with or without MCM6 over-expression plasmid were placed in 96-well plates. A total of 10 nM E2 and 1 μ M P4 were added to transfected HESCs and incubated for 24 h, 48 h, and 72 h. For detecting the cell viability, 10 μ l CCK-8 reagent (Seyotin, Guangzhou, China) was administrated to HESCs. After 2–3 h of incubation, the OD value (450 nm) was tested under a microplate reader (Bio-Tek Instruments Inc, Winooski, VT, USA).

Cell Cycle Distribution Analysis

Cell distribution of HESCs was examined after treatment with or without E2P4 for 72 h. After trypsinization, HESCs were collected. After washing with PBS, HESCs were incubated with ethanol (70%) at -20 °C for 12 h. Staining of HESCs was conducted using the Cell Cycle and Apoptosis Analysis kit (Beyotime, Shanghai China). Finally, HESCs were detected under a flow cytometry (BD Biosciences, Sao Jose, CA, USA).

Immunohistochemistry Staining

Paraffin-sectioned tissues of endometrial specimens were dewaxed with xylene and dehydrated. Antigen retrieval was performed by microwave heating the slides in sodium citrate buffer for 15 min. Slides were then put into 3% H₂O₂ and incubated for 15 min. After two washes with PBS, anti-MCM6 antibody was applied to the slides followed by biotinylated secondary antibodies. The color examination was undertaken using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). After counterstaining with hematoxylin, MCM6 expression was visualized under an Olympus BX-51 microscope (Olympus, Tokyo, Japan). The relative expression of MCM6 was quantified using histologic scoring (H-score) analysis.

Immunofluorescence Staining

HESCs were trypsinized and collected. HESCs (1×10^5) were seeded on glass coverslips. After overnight incubation, HESCs were treated with or without E2P4 for 3 days. After fixing with 4% paraformaldehyde for 15 min, HESCs were rinsed three times with PBS. HESCs were blocked with a blocking buffer (2% BSA) for half an hour. HESCs were probed with anti-MCM6 antibody (1:100 dilution) overnight at 4 °C. Following rinsing with PBS, HESCs were probed with secondary antibodies conjugated with Alexa Fluor488

(1:500 dilution). The incubation lasted for 1 h away from light. Nuclear staining was conducted with the DAPI solution. Cell images were taken with a Carl Zeiss LSM710 microscope (Carl Zeiss, Jena, Germany).

Western blotting

HESCs were washed, trypsinized, and harvested. HESCs were lysed in RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China). Endometrial tissues in the proliferative and secretion endometrium were homogenized using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China). The lysates of HESCs or tissues were maintained at 4 °C for 20 min, and then cell debris was precipitated by centrifugation. The supernatant was removed to a new tube. Detection of protein concentration was performed using a BCA kit (Millipore, Bedford, MA, USA). Twenty micrograms of each sample was loaded on SDS-PAGE gel. The separated proteins were transferred onto PVDF membranes (Millipore, Bedford, MA, USA). The transferred membranes were incubated with non-fat milk (5%). Next, membranes were probed with primary antibodies for 12 h at 4 °C and probed with secondary antibodies for 1.5 h. Protein detection was performed using an ECL detection kit (Seyotin, Guangzhou, China). The relative protein expression was quantified using Image J software.

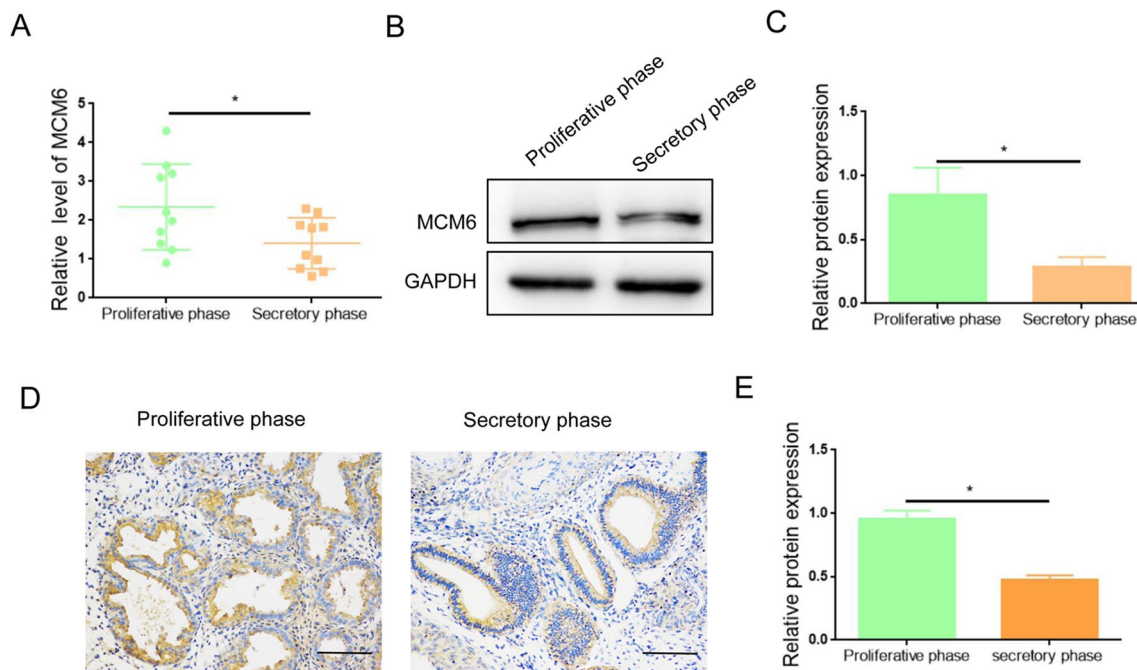


Fig. 1 MCM6 level is downregulated in the secretory endometrium. **A** The mRNA expression levels of MCM6 in endometrium ($n = 10$) were analyzed by qRT-PCR. **B** MCM6 protein content in the endometrium was analyzed by Western blotting. **C** Quantification of MCM6

expression shown in **B**. **D** MCM6 protein content in the endometrium was examined by immunohistochemistry. **E** Quantification of MCM6 expression shown in **D**. * $P < 0.05$

Statistical Analysis

Experiment data were represented as mean \pm SD. Graph-Pad Prism 6 software (GraphPad) was utilized for statistical analysis. Differences were accessed with Student *T*-test (two groups) or one-way ANOVA (more than two groups). Statistically, significance was considered when the *P* value was lower than 0.05.

Results

MCM6 Expression Is Decreased in Secretory Endometrial Specimens

To study the relevance of MCM6 in decidualization, we examined the expression of MCM6 in the proliferative and secretory endometrial tissues. qRT-PCR result revealed a

significant decrease in MCM6 mRNA level in the secretory tissues in comparison with the proliferative tissues (Fig. 1A). In agreement with this data, Western blotting and immunohistochemistry analysis revealed that MCM6 level in secretory samples was decreased as compared with proliferative samples (Fig. 1B–E). Therefore, these data indicated that MCM6 expression is downregulated in the secretory endometrial tissues.

E2P4 Suppresses MCM6 Expression in HESCs

To address the role of MCM6 in decidualization, we in vitro established in HESCs by E2P4 exposure, as reported previously [19]. To confirm the success of the establishment, we determined the changes of two major mediators of decidualization, insulin-like growth factor-binding protein 1 (IGFBP-1) and prolactin (PRL) [20]. As shown in Fig. 2A–C, IGFBP-1 and PRL expressions were elevated

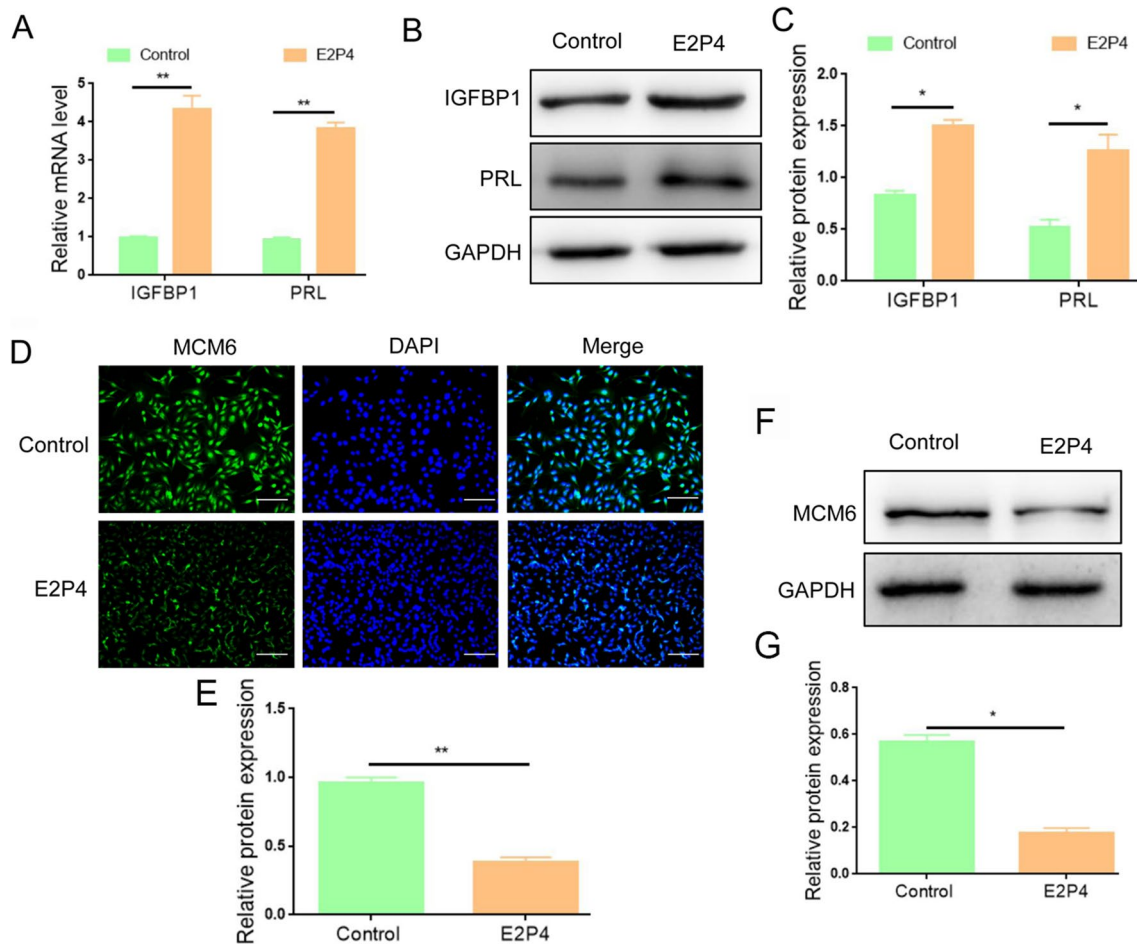


Fig. 2 MCM6 expression is decreased in E2P4-treated HESCs. **A** IGFBP1 and PRL mRNA levels in HESCs were analyzed by qRT-PCR. **B** IGFBP1 and PRL protein levels in HESCs were tested by Western blotting. **C** Quantification of IGFBP1 and PRL expression shown in **B**. **D** MCM6 protein levels in HESCs were detected by

immunofluorescence staining. **E** Quantification of MCM6 expression shown in **D**. **F** MCM6 protein content in HESCs was tested by Western blotting. **G** Quantification of MCM6 expression shown in **F**. **P* < 0.05; ***P* < 0.01

at both mRNA level and protein level in HESCs treated with E2P4. Compared with control cells, increased MCM6 intensity was observed in E2P4-treated cells, as analyzed by immunofluorescence staining (Fig. 2D and E). In consistent with these results, MCM6 protein level and mRNA levels were downregulated in HESCs following E2P4 treatment (Fig. 2F, G, and Fig. S1).

E2P4 Triggers G1-Phase Cell Cycle Arrest in HESCs

The stromal cell proliferation is crucial for the process of decidualization. Thus, we assess the regulation of E2P4 in the growth of HESCs. The viability of E2P4-treated HESCs was markedly reduced in comparison with control cells (Fig. 3A). As cell cycle regulation is important even during decidualization, we determined whether E2P4 treatment could disturb the distribution of the cell cycle. We observed an increased number of cells accumulated at G1 cell cycle phase following E2P4 administration (Fig. 3B and C). Moreover, the mRNA and protein levels of G1 cell cycle phase-associated regulators (p21, cyclin-dependent kinase 2 (CDK2), Cyclin D1, Cyclin E1) were reduced in E2P4-exposed HESCs (Fig. 3D–F, Fig. S2). Together, the above results indicated that E2P4 treatment triggers cell cycle arrest in the G1-phase of HESCs.

MCM6 Suppresses E2P4-Induced Decidualization

To investigate the function of MCM6 in the decidualization of HESCs, ectopic expression of MCM6 was carried out in HESCs by transfecting the MCM6 overexpression plasmid. Western blotting confirmed an increase in the MCM6 protein level in HESCs (Fig. 4A and B). Next, we assessed the requirement of MCM6 in the growth of HESCs. E2P4 administration decreased the growth of HESCs, and this effect was counteracted by ectopic expression of MCM6 (Fig. 4C). Moreover, overexpression of MCM6 counteracted the promoting effect of E2P4 on the mRNA levels of PRL and IGFBP1 (Fig. S3). Additionally, while E2P4 treatment caused an elevation in the G1 phase population, overexpression of MCM6 reduced the cell number in G1 phase (Fig. 4D and E). In agreement with the enrollment of MCM6 on cell cycle promotion, MCM6 overexpression increased the abundance of CDK2, Cyclin D1, and Cyclin E1, which was decreased by E2P4 (Fig. 4F–H). Collectively, these data indicated that MCM6 at least partially suppresses E2P4-induced decidualization in HESCs by regulating cell proliferation and cell cycle progression.

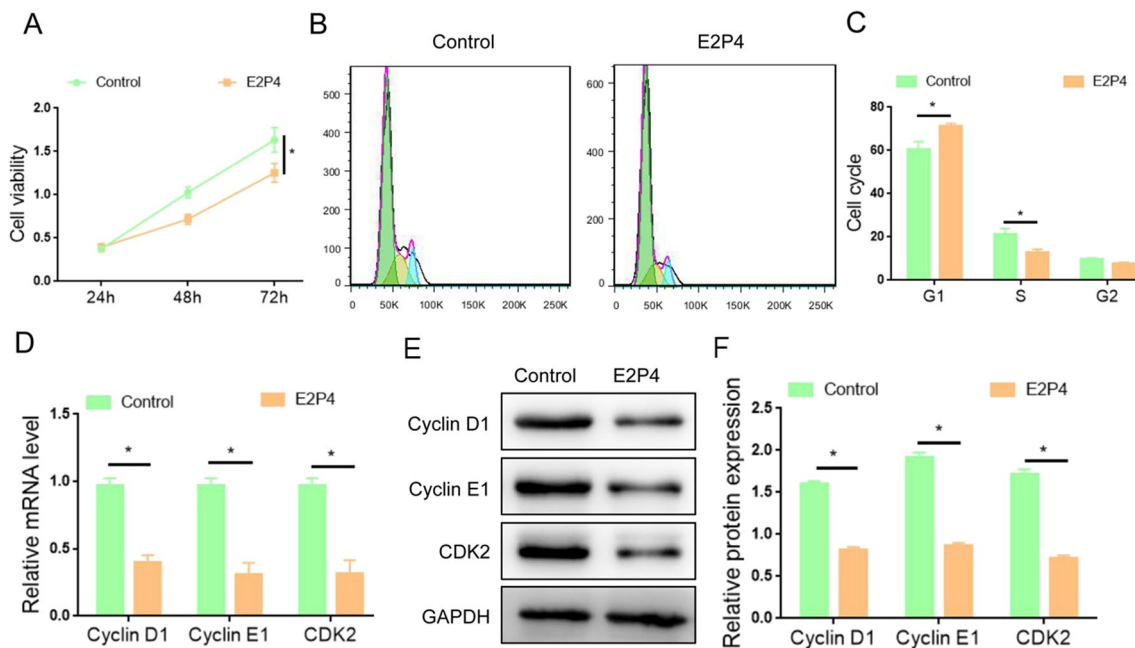


Fig. 3 E2P4 treatment blocks HESC proliferation and causes G1 phase arrest. **A** The proliferative activity of HESCs that were treated with or without E2P4 was analyzed using the CCK-8 assay. **B** The cell cycle distribution of HESCs that were treated with or without E2P4 was determined by flow cytometry. **C** Quantification of cell cycle distribution as shown in **B**. **D** The mRNA levels of G1 phase-

associated genes in HESCs that were treated with or without E2P4 were analyzed using qRT-PCR. **E** The protein levels of G1 phase-associated genes in HESCs that were treated with or without E2P4 were analyzed using Western blotting. **F** Quantification of the expression levels of G1 phase-associated genes as shown in **D**. * $P < 0.05$, ** $P < 0.01$

MCM6 Regulates E2P4-Induced Decidualization via the ERK Pathway

Next, we explored the molecular mechanism by which MCM6 regulates E2P4-induced decidualization. Given the important role of the ERK pathway in decidualization and MCM6 can crosstalk with ERK signaling [17, 18], we investigated whether MCM6 controls decidualization via the ERK pathway. Exposure of E2P4 to HESCs decreased the phosphorylation of mitogen-activated protein kinase 2 (MEK2) and ERK, and overexpression of MCM6 restored p-MEK2 and p-ERK levels (Fig. 5A and B). Administration of ERK agonist Ro 67–7476 elevated the levels of p-ERK and MCM6, which were suppressed by E2P4 (Fig. 5C and D). These results confirmed a feedback circuit between MCM6 and ERK. In addition, while E2P4 exposure elevated the cell accumulation at G1 phase, treatment of Ro 67–7476

reversed this effect (Fig. 5G and F). Consistently, the inhibition of E2P4 on CDK2, Cyclin D1, and Cyclin E1 content was abolished by Ro 67–7476 administration (Fig. 5G–I). Together, these data indicated that MCM6 partially regulates decidualization via cross-talking with ERK signaling.

Discussion

Proper decidualization is crucial for pregnancy success and improving apprehension of the regulatory mechanisms of this process will facilitate access to this goal. Our data revealed that MCM6 abundance was decreased in both the secretory endometrial specimens and E2P4-treated HESCs. Our data further revealed that MCM6 modulates E2P4-induced decidualization in vitro by cross-talking with ERK signaling.

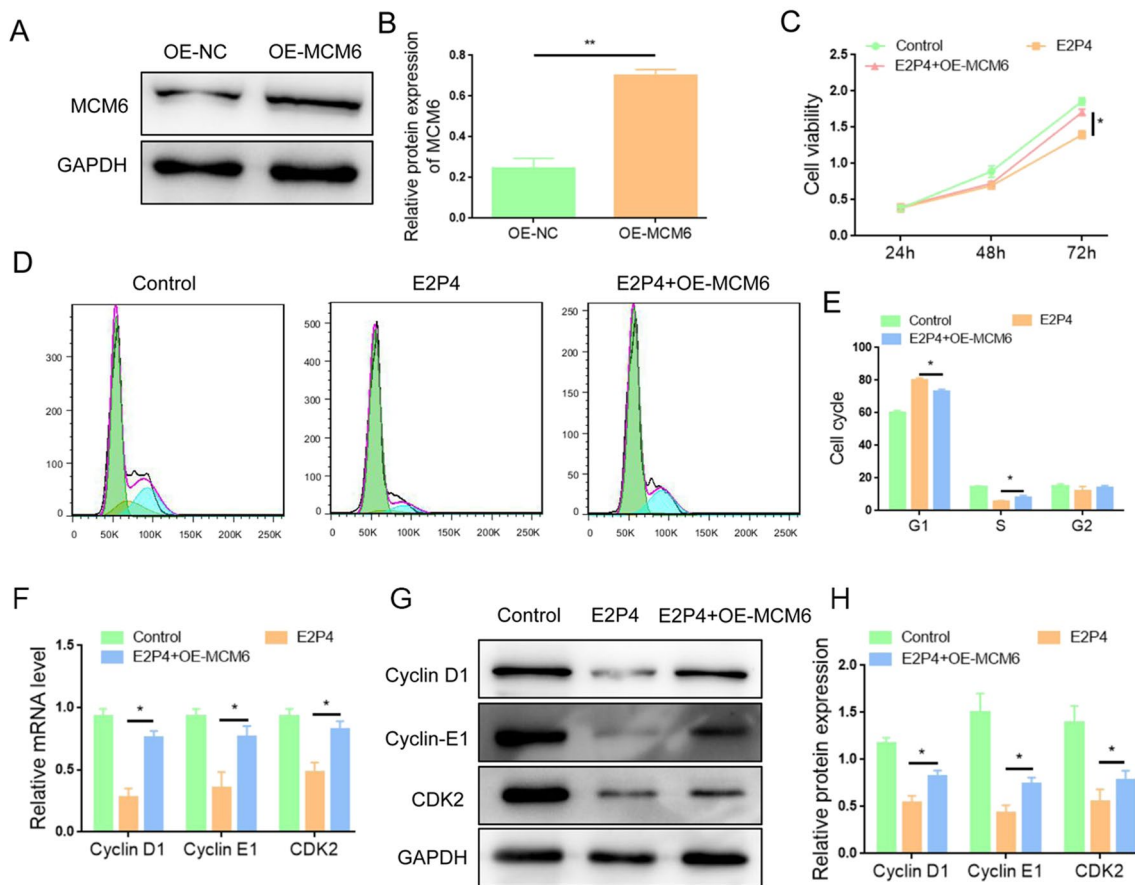


Fig. 4 Ectopic expression of MCM6 reverses the effects of E2P4 on HESCs. **A** The content of MCM6 in HESCs was detected after transfection with MCM6 overexpression plasmid (OE-MCM6) or control vector (OE-NC). **B** Quantification of MCM6 expression as shown in **A**. **C** Overexpression of MCM6 elevated the cell viability that was repressed by E2P4 exposure in HESCs. **D** Transfection of MCM6 counteracted the action of E2P4 on the distribution of the cell cycle in HESCs. **E** Quantification of cell cycle distribution as shown in **D**.

F The mRNA levels of G1 phase-associated genes in different treatment groups (Control, E2P4, E2P4 + OE-MCM6) were addressed by qRT-PCR. **G** The protein expressions of G1 phase-associated genes in different treatment groups (Control, E2P4, E2P4 + OE-MCM6) were addressed by Western blotting. **H** Quantification of the expression levels of G1 phase-associated genes as shown in **G**. * $P < 0.05$, ** $P < 0.01$

The process of decidualization is stringently modulated by the proliferation and differentiation of stromal cells, in which many cell cycle regulatory factors are involved [19]. The cell cycle progression is precisely controlled at two cell cycle checkpoints, the G1 checkpoint and the G2/M checkpoint, of which the G1 checkpoint is crucial [21]. Cyclin-dependent kinases, as well as their inhibitors, are key controllers of the cell cycle [22]. Among these regulators, Cyclin D1-3 are well-known to modulate the G1 phase. At the G1 phase, increased cyclin D proteins bind to CDK4/6 and promote the G1-S phase transition [23]. Cyclins E1-2 are expressed during the late G1 phase, which interact and activate CDK2, triggering the S phase entry as well. In the in vitro decidualization model, we found that the cell population of HESCs in G1 phase was significantly increased, which was accompanied with decreased expressions of

cyclin D1/E1 and CDK2, indicating an arrest at G1 phase in this model.

It has been shown that treatment with P4 suppresses the binding of MCM to chromatin and thus counteracts E2-stimulated DNA replication [24], implying an implication of MCM proteins in E2P4-mediated cell cycle progression. MCM6 is a key regulator in cell proliferation, which controls DNA replication, as well as cell cycle [10, 25]. It has been reported that silencing of MCM6 blocks G1/S progression, leading to cell growth suppression in neuroblastoma cells [10]. Dabral et al. have also reported that MCM6 associates with latency-associated nuclear antigen at the G1/S cell cycle phase and thus supports DNA replication in herpesvirus [26]. Consistently, we found that overexpression of MCM6 triggered the G1

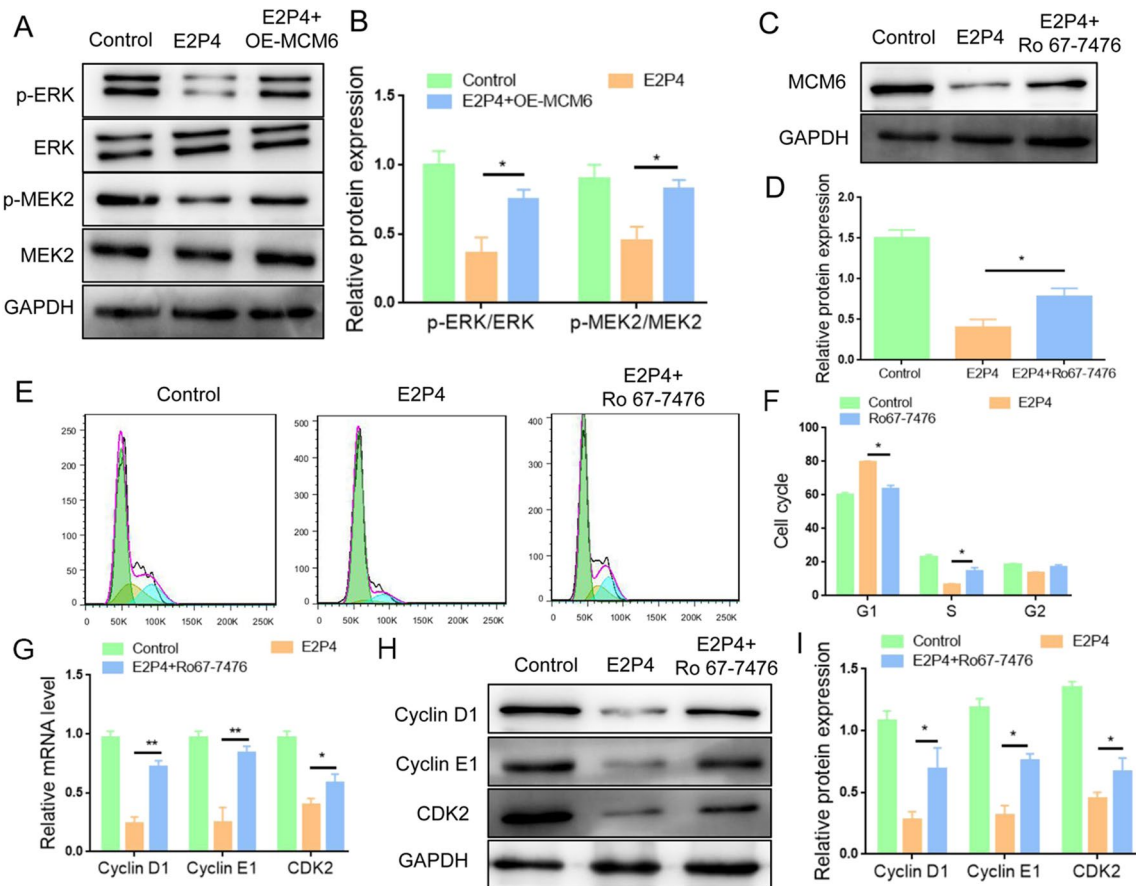


Fig. 5 MCM6/ERK signaling contributes to E2P4-induced decidualization. **A** Overexpression of MCM6 restored the phosphorylation of MEK and ERK in E2P4-treated HESCs. **B** Quantification of the phosphorylation levels of MEK and ERK as shown in **A**. **C** MCM6 protein levels in HESCs were tested by Western blotting after treatment with E2 (10 nM)/P4 (1 μ M) for 24 h alone or together with Ro 67-7476 (1 μ M) for 10 min. **D** Quantification of MCM6 expression as shown in Fig. 3C. **E** Cell cycle distribution in different treatment groups after treatment with E2 (10 nM)/P4 (1 μ M) for 24 h alone or

together with Ro 67-7476 (1 μ M) for 10 min. **F** Quantification of cell cycle distribution as shown in **E**. **G** The mRNA levels of G1 phase-associated genes in HESCs were quantified by qRT-PCR after treatment with E2 (10 nM)/P4 (1 μ M) for 24 h alone or together with Ro 67-7476 (1 μ M) for 10 min. **H** The protein levels of G1 phase-associated genes in HESCs were quantified by Western blotting after treatment with E2 (10 nM)/P4 (1 μ M) for 24 h alone or together with Ro 67-7476 (1 μ M) for 10 min. **I** Quantification of the expression levels of G1 phase-associated genes as shown in **H**. * $P < 0.05$, ** $P < 0.01$

phase progression and thus reversed that inhibitory effect of E2P4 on cell viability of HESCs.

ERK pathway is well-known for modulating cell growth, survival, as well as cell cycle [27]. ERK pathway has also been reported to play a critical role in decidualization. It has been shown that heparin-binding EGF-like growth factor (HB-EGF) modulates the content of putative serine protease 56 (Prss56) at the implantation site by ERK signaling during mouse decidualization [28]. Zhou et al. have reported that fibroblast growth factor 7 (FGF7) accelerates endometrial stromal cell proliferation via ERK and Jun N-terminal Kinase (JNK) pathways [29]. Previous studies have indicated there is a feedback loop between MCM6 and ERK signaling [17, 18, 25, 30]. Consistently, we found that ectopic expression of MCM6 restored the phosphorylation of ERK inhibited by E2P4, and treatment with ERK pathway agonist Ro 67–7476 increased the levels of MCM6 suppressed by E2P4. Moreover, treatment with Ro 67–7476 reversed the effects induced by E2P4 exposure. Hence, our data implied that MCM6/ERK signaling plays a vital role in E2P4-mediated decidualization.

In conclusion, our data reveal that MCM6 suppresses E2P4-induced decidualization in HESCs via its crosstalk with ERK signaling. The findings of this study highlight that MCM6 could be a potential target for meliorating uterine receptivity.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s43032-024-01463-5>

Author Contribution (I) Conception and design: YJ and XY; (II) administrative support: SY; (III) provision of study materials or patients: ML; (IV) collection and assembly of data: XY and YS; (V) data analysis and interpretation: YJ and HZ; (VI) manuscript writing: all authors; (VII) final approval of manuscript: all authors.

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Data Availability Due to the nature of this research, participants of this study did not agree for their data to be shared publicly, so supporting data is not available.

Declarations

Consent to Participate All patients consent to be enrolled in the work.

Conflict of Interest The authors declare no competing interests.

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