ABSTRACT

Uterine stromal cells undergo extensive proliferation and differentiation during postimplantation development, a process known as decidualization. While a range of signaling molecules have been demonstrated to play essential roles in this event, its potential epigenetic regulatory mechanisms remained largely unknown. Retinoblastoma binding protein 7 (Rbbp7) is a protein reported as a core component of many histone modification and chromatin remodeling complexes. In the present study, our in situ hybridization and immunochemistry analysis first revealed a spatiotemporal expression of Rbbp7 in the uterus during the periimplantation period. Observations of remarkable induction of Rbbp7 expression in uterine stromal cells in response to progesterone-nuclear receptor PR signaling pointed toward to its potential physiological significance during postimplantation uterine development. Employing stealth RNA knockdown approach combined with primary murine uterine stromal cell culture and in vitro induced decidualization model, we further demonstrated that Rbbp7 silencing compromises stromal cell decidualization via attenuating histone H4 acetylation and cyclin D3 expression. The results collectively suggested that Rbbp7 is a potentially functional player regulating normal histone acetylation modification and cyclin D3 expression in stromal cells during postimplantation decidual development.

Keywords: Rbbp7, uterine stroma, decidualization, cyclin D3, acetylation

INTRODUCTION

Pregnancy is a complex process that comprises discrete events including implantation, decidualization, placentation and birth of offspring, and the success of each is essential to normal pregnancy [1]. After implantation, uterine stromal cells surrounding the implanting
embryo undergo extensive proliferation and differentiation in mice, and this process is known as decidualization [2]. This uterine stromal-decidual transformation primarily directed by progesterone and estrogen is critical for supporting embryo nutrition and other important aspects before a mature placenta is formed [3, 4]. Due to ethical restrictions on pregnancy-related research in women, mouse model has been widely used and advanced the field further by identifying genes crucial for decidualization [1, 5]. The feature of decidualization in mice is associated with the formation of multinucleate and giant cells that are developed through a unique process requiring transition from the mitotic cell cycle to an endoreduplication cycle, when cells undergo a repeated round of DNA replication but without successive cell division [6]. Although many molecules and signaling pathways such as transcription factors, morphogens, cytokines, cell cycle regulators have been shown to play essential roles during decidual development [1], knowledge on the epigenetic regulatory machinery governing uterine decidualization remained limited.

Emerging evidence suggests that histone modification involves in a wide ranges of developmental and pathophysiological processes, including in early pregnancy events [7-10]. As generally accepted, post-translational modification of histones converts unique regions of chromatin into transcriptionally active or inactive status. There are four kinds of core histones, H2A, H2B, H3 and H4, the amino-terminal tails of which provide the sites for acetylation, methylation, phosphorylation and ADP-ribosylation [11]. Among these modifications of histones, the most well studied is the acetylation on lysine residues in the amino-terminal tail of the histones [12]. In the early 1960s, Vincent Allfrey and his colleagues reported the identification of histone acetylation and proposed a regulatory role of this modification in transcription regulation [13]. Then two classes of enzymes involved in regulating acetylation status of histones were identified: histone acetyltransferases (HATs) and histone deacetylases (HDACs) [12]. Wolffe and Pruss reported that hyperacetylation of core histones correlates with gene activation, while hypoacetylation of core histones correlates with gene repression [14]. In this respect, there were a few studies reported that inhibition of histone deacetylation by HDACs inhibitors, such as trichostatin A (TSA), can accelerate the progression of human stromal cell differentiation [7]. Moreover, some transcription factors and signaling molecules have been shown to affect decidualization through altering the state of histone acetylation [15, 16]. For example, Tamura et al. explored that CCAAT/enhancer binding protein β (CEBPB) can regulate the expression of insulin-like growth factor binding protein 1 (IGFBP1) and prolactin (PRL), two well-known markers of human stromal cell decidualization, by altering acetylation of H3K27 in their promoters [15]. Despite that histone hyperacetylation is correlated with human stromal-decidual transformation, it remained largely unknown whether histone hypoacetylation would affect decidualization.

Previous studies have demonstrated that evolutionarily conserved Rbbp7 (also called RbAp46) is a functional member of several histone modification and chromatin remodeling complexes, including histone acetylation complex, histone deacetylation complex (such as NuRD and Sin3), polycomb repressive complex 2 (PRC2) [17, 18]. It is generally accepted as far that Rbbp7 can bind to H4 histone and facilitates H4 interaction with chaperones and other
molecules [17, 19], thus involves in regulating cell proliferation and differentiation in variety species [20-22]. RBBP7 was first identified as a major associated protein of the retinoblastoma protein (Rb) in Hela cells using Rb affinity column and can interact with Rb in vitro and in vivo [23, 24]. In mice, higher mRNA level of Rbbp7 was observed in the uterus, gonad, brain, thymus, kidney than other tissues [24-26]. RBBP7 was reported to be highly expressed in human oocytes through microarray approach, suggesting its potential function during oocyte maturation [27]. Knockdown of Rbbp7 in mouse oocyte affects meiotic progression and chromosome segregation possibly by perturbing histone acetylation status [28]. Moreover, dysregulated RBBP7 expression was reported to contribute to estrogen-stimulated breast cancer [29, 30], pure ductal carcinoma in situ [31] and brain tumor [32]. However, it remained to explore regarding the pathophysiological significance of Rbbp7 in decidualization during early pregnancy.

To address this issue, in the present investigation, we first analyzed the spatiotemporal expression of Rbbp7 in the uterus at periimplantation. Employing stealth RNA knockdown approach combined with in vitro induced stromal cell decidualization model, we further demonstrated that Rbbp7 silencing compromises stromal cell decidualization via attenuating histone acetylation and cyclin D3 expression.

**MATERIALS AND METHODS**

**Reagents and antibodies**

All reagents and antibodies including Diethanolamine (Sigma), Phosphatase substrate (Sigma), BCIP/NBT Color Development Substrate (Promega), C646 (Selleck), anti-Rbbp7 (Abcam), anti-Vimentin (Epitomics), anti-BrdU (Abcam), anti-cyclin D3 (Cell Signaling Technology), anti-β-actin (ABclonal technology), anti-H4 (Abcam), anti-acH4K5 (Abcam), anti-acH4K8 (Abcam), anti-acH4K12 (Abcam), HRP-conjugated secondary antibody (Beijing ZhongShan Biotechnology), were purchased from the respective companies.

**Mice and treatments**

Mice were housed in Institutional Animal Care Facility of Institute of Zoology according to institutional guidelines for laboratory animals. Mice of CD1 background (8-weeks old) were purchased from the Vital River Laboratory Animal Technology Co. Ltd. PR null mutant mice were generated as described previously [33], and were kindly provided by Dr. Francesco DeMayo. The female mice with the vaginal plug after caged with the fertile or vasectomized male on the next day morning were considered as day 1 of pregnancy or pseudopregnancy, respectively. Artificial decidualization was induced by intraluminal oil perfusion in day 4 morning in pseudopregnant females. To test the ovarian hormonal influence on uterine Rbbp7 expression, wild-type and PR+/− mice were ovariectomized and rested for 7 days [34-36], then were injected with oil, estradiol-17β (E2, 100 ng/mouse), progesterone (P4, 2 mg/mouse), or a combination of E2 and P4. Mice were killed at indicated times, and uteri processed for immunostaining and mRNA analysis by quantitative RT-PCR.

**Primary uterine stromal cell culture**
Murine primary uterine endometrial stromal cells (mESCs) were isolated and cultured as previously described with some modifications [37]. The uterus of pseudopregnant mouse on day 4 was cut into small pieces (3-4 mm). Those tissue pieces were first digested in Hanks’ balanced salt solution (HBSS) containing 6 mg/ml dispase (Gibco) and 25 mg/ml trypsin (Sigma), and then incubated in HBSS containing 0.5 mg/ml collagenase (Gibco). The digested cells were passed through a 70 µm filter to obtain the stromal cells. Cells were plated at 5×10^5 cells per 60 mm dish or the corresponding numbers according to dish area, cultured with phenol red-free Dulbecco’s Modified Eagle’s Medium and Ham’s F-12 nutrient mixture (1:1, DMEM/F12, Gibco) containing 10% charcoal-stripped fetal bovine serum (C-FBS, Biological Industries) and antibiotics. 2h later, the medium was replaced with fresh medium and cultured overnight. On next morning, the medium was replaced with DMEM/F12 containing 1% C-FBS, E2 (10 nM) and P4 (1 µM) and antibiotic to induce decidualization.

**Stealth RNA transfection**

Stealth RNA corresponding to mouse Rbbp7 (AUCAGAUGCUCUGG AGAUGCCCA) was designed and synthesized by Invitrogen. The stealth RNA or control siRNA was transfected into mESCs following the protocol of Lipofectamine RNAiMAX (Invitrogen). Briefly, 75 pmol of stealth RNA was used to form complexes with RNAiMAX in phenol red-free Opti-MEM I Reduced Serum Medium (Gibco) and dispersed into 6-well cell culture plate.

**Western blotting**

Protein extracts of cells were dissolved in RIPA lysis buffer containing proteinase inhibitor. After determining the protein concentration, protein lysates were separated by SDS-PAGE, and then were transferred to PVDF membranes, blocked with 5% skimmed milk. After incubating with primary antibodies overnight, the membranes were incubated with HRP-linked secondary antibodies. Bands on the membranes were detected using SuperSignal West Pico (Thermo Scientific).

**In situ hybridization**

In situ hybridization with isotopes or digoxygenin was performed as previously described [38]. Mouse-specific cRNA probe for Rbbp7 was used for hybridization. Section hybridized with the sense probe served as a negative control.

**Immunostaining**

Immunohistochemistry was performed in 5 µm paraffin-embedded sections. Tissues were fixed in 10% neutral buffered formalin or 4% paraformaldehyde, dehydrated via graded ethanol solutions, and embedded in paraffin. The procedure of immunohistochemistry analysis was described previously [39]. Briefly, the sections were subjected to a high-pressure antigen retrieval technique and the endogenous peroxidase activity was quenched. After blocking with a blocking serum, sections were incubated overnight with primary antibodies. The sections were incubated with HRP-conjugated secondary antibodies next day. Immunofluorescence was
performed with cells on coverslips that were fixed by 4% paraformaldehyde. After washing with phosphate buffer saline (PBS) and punching with Triton X-100-PBS, cells were blocked in 0.5% bovine serum albumin and then incubated with primary antibodies overnight. On next day, cells were incubated with secondary antibodies and the nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI).

**RNA extraction and real-time polymerase chain reaction (PCR)**

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed as described [37]. Total RNA was isolated from uterine tissues or cells with TRIzol reagent following the manufacturer’s protocol, and 1-3µg of RNA was used to synthesize cDNA. The expression levels of different genes were validated by real-time PCR analysis using SYBR green method in ABI 7500 according to manufacturer’s instructions (Applied Biosystems). All primers for real-time PCR were listed in Supplemental Table S1 (Supplemental Data are available online at www.biolreprod.org).

**Cell proliferation assays**

The experiment with CellTiter 96 AQueous One Solution Cell Proliferation Assay was performed following the protocol of the manual. Briefly, stromal cells were plated in 96-well plate and starved overnight, then culture medium was replaced by DMEM/F12 containing 1% C-FBS and antibiotic to recover cell proliferation. At 24h, 48h and 96h, reagent (MTS) was added to culture medium and the plate was incubated at incubator for 3 hours, then the supernatant was pipetted to detect its absorbance at 490nm. BrdU incorporation was performed after cells being starved overnight, and antibody against BrdU was used for immunofluorescence analysis.

**Statistical analysis**

All data are presented as mean ± SEM. Each experiment included at least three independent samples. Comparison between two groups was made by unpaired Student’s two-tailed t-test, and comparisons among three groups or more were made by one way ANOVA test. $P<0.05$ was considered to indicate a significant result.

**RESULTS**

*Rbpb7 is spatiotemporally expressed in the periimplantation uterus*

To reveal the function of Rbpb7 in early pregnancy events, we first assessed the spatiotemporal uterine expression of *Rbpb7* by in situ hybridization and immunohistochemistry analysis. As shown in Figure 1, *Rbpb7* at both mRNA and protein level was mainly expressed in epithelium cells on day 1 of pregnancy under a profound influence of preovulatory estrogen surge. On day 4, with an increased progesterone secretion from the newly formed corpus luteum, its expression was detected in stromal cells. This dynamic shift of Rbpb7 expression from the luminal epithelium on day 1 of pregnancy to the stroma on day 4 indicated that preimplantation estrogen and progesterone may regulate the uterine Rbpb7 expression. With
the onset of embryo attachment reaction, Rbbp7 was extensively expressed in the stromal cells surrounding the implanting blastocyst on day 5. On day 6 and beyond, its major expression in uterine stroma gradually shifted from the antimesometrial pole to the mesometrial pole that was accompanied with the progression of decidualization from day 6 to day 8 (Figure 1A and B). It was worthy to note that Rbbp7 was also expressed in the decidualized stromal cells exhibiting large or multiple nuclei (Figure 1B), pointing toward its potential function during postimplantation uterine decidualization. On day 8, Rbbp7 was not only strongly expressed in stromal cells, but also in vascular endothelial cells and immunocytes, such as the DBA-lectin positive uNK cells (Supplemental Figure S1), which also undergone extensive proliferation and differentiation during decidualization. In addition, we also found the expression of Rbbp7 in postimplantation embryo, indicated its potential function during postimplantation embryo development.

**Progesterone via nuclear PR receptor induces Rbbp7 expression in stromal cells**

To address the influence of progesterone and estrogen on uterine Rbbp7 expression, we employed ovariectomized mouse model injected with E2, P4, or E2 plus P4. As illustrated in Figure 2A, both E2 and/or P4 can significantly upregulate Rbbp7 mRNA expression. Subsequent immunostaining revealed a cell-specific expression of Rbbp7 in response to ovarian steroid hormones. While Rbbp7 expression was sustained in the luminal and glandular epithelium even in the oil group, estrogen treatment tended to increase Rbbp7 expression in the epithelial layer, but elicited no remarkable effects on stromal Rbbp7 expression (Figure 2B). By contrast, progesterone either alone or together with estrogen significantly upregulated Rbbp7 expression in stromal cells (Figure 2B). This facilitative activity of progesterone on stromal Rbbp7 expression largely depended on nuclear PR receptors, since progesterone failed to induce Rbbp7 expression in PR null mutant stromal cells (Figure 2C).

**Rbbp7 silencing via stealth RNA compromises uterine stromal cell proliferation and decidualization**

To stabilize the basis of Rbbp7’s potential involvement in decidualization, we employed an artificially induced decidualization model and further analyzed Rbbp7 expression in deciduoma. As illustrated in Figure 3A and B, while Rbbp7 expression was mainly detected in the luminal and glandular epithelium in the non-oil infused uterine horn, its expression was dramatically induced in the decidualizing stromal cells after intraluminal oil infusion.

We then employed mouse primary uterine endometrial stromal cell (mESC) in vitro decidualization model to further investigate the function of Rbbp7 during decidualization. As shown in Supplemental Figure S2A, the purity of mESCs harvested from day 4 pseudopregnant females was confirmed by immunofluorescence staining of vimentin, a stromal cell marker [40]. Moreover, stromal decidualization can be induced in culture in response to cotreatment of estrogen and progesterone, as verified by increased mRNA expression of Prl8a2 (prolactin family 8, subfamily a, member 2, previously known as Dtprp) (Supplemental Figure S2B), a well-known marker of decidual cells [41, 42]. Immunofluorescence staining revealed an obvious
expression and nuclear localization of Rbbp7 in mESCs in culture (Figure 4A), while this expression at both mRNA and protein level can be significantly downregulated via transfection with stealth RNA selectively targeting Rbbp7 (Figure 4B-D).

During endometrial decidualization, uterine stromal cells first undergo extensive proliferation and then differentiation. To investigate whether Rbbp7 play a role in proliferation of uterine stromal cells, we performed cell proliferation assay and BrdU incorporation experiment (Figure 4E-G). The results showed that Rbbp7 silencing compromised stromal cell proliferation. Meanwhile, the downregulation of Rbbp7 greatly hampered the decidualization process of mESCs in culture, since the expression of Dtprp was dramatically reduced in stealth RNA transfected stromal cells (Figure 4H). This impaired mESC decidualization upon Rbbp7 silencing was further confirmed by the observation of reduced alkaline phosphatase activity (Figure 4I and J), which is another typical biochemical marker of differentiated stromal cells [43, 44].

**Rbbp7 silencing attenuates uterine stromal cell histone acetylation and cyclin D3 expression.**

To explore the causes of impaired decidualization in mESCs upon Rbbp7 knockdown, we next analyzed the expression of various stromal cell decidualization associated molecules as well as cell cycle-related molecules. As shown in Supplemental Figure S3, all tested genes including Bmp2, Wnt4, Cebpβ, Cdk1, Cdk2, Cdk4, Ccnb1, Ccne1 and Cdkn1a (also known as P21) showed comparable expression even in Rbbp7 knockdown group. Interestingly, we noted a dramatically reduced both mRNA and protein expression of cyclin D3 during in vitro proliferation and decidualization (Figure 5B-F). The colocalization analysis of cyclin D3 and Rbbp7 revealed while Rbbp7 exhibited a border expression pattern in the postimplantation chamber on days 6 and 8 of pregnancy, most of cyclin D3-expressing cells also showed Rbbp7 expression (Figure 5A), which is consistent with the results from the in vitro cultured stroma cells (Supplemental Figure S4). This observation suggested that cyclin D3 could be a targeting gene of Rbbp7 mediated histone modification. We thus surmised that histone acetylation status could be altered in mESCs upon Rbbp7 silencing by stealth RNA.

To verify our speculation, we analyzed the protein level of acetylated histones at common acetylation sites of H4 by western blotting, and observed that the level of histone acetylation was generally reduced in Rbbp7 knockdown group compared to control group (Figure 6A, B), which suggested that Rbbp7 may involve in regulating histone acetylation and thus ensure normal stromal-decidual transformation. This notion was further evidenced by our findings that treatment of mESCs with histone acetyltransferases inhibitor, C646, largely reduced the expression decidual marker gene Dtprp (Figure 6C). It was conceivable that a hypoacetylation status of histone is associated with impaired decidualization. Indeed, we observed a dramatic reduction of acH4K5 level upon C646 treatment in mESCs (Figure 6D). Most interestingly, this declined H4K5 acetylation was coincident with a remarkably reduced cyclin D3 expression (Figure 6E), similar to that after Rbbp7 silencing. These findings collectively suggested that Rbbp7 silencing compromises stromal cell decidualization probably via attenuating histone acetylation and cyclin D3 expression.
DISCUSSION

It is believed that decidua is responsible for providing nutrition to embryo and forming barrier to prevent uncontrolled trophoblast invasion before the formation of a mature placenta. Therefore, a normally developed decidua is critical for successful pregnancy [3]. During the past few decades, a wealth of signaling pathways and key regulatory molecules have been identified to be essential for normal postimplantation decidual development. It remained to be paid more attention regarding the potential epigenetic regulatory mechanisms in this early pregnancy event. In this respect, we demonstrated that Rbbp7, a histone binding component of many histone modification complexes, was spatiotemporally expressed in the periimplantation uterus. Moreover, we provided herein novel evidence that Rbbp7 is essential for normal histone acylation modification and cyclin D3 expression in mESCs, ensuring normal stromal decidualization.

Ovarian estrogen and progesterone are the primary determinants governing normal uterine functions during implantation and decidualization [3, 5]. We observed that Rbbp7 expression in differential uterine cell-types can be profoundly influenced by estrogen and progesterone. While estrogen tended to exert less influence on stromal Rbbp7 expression, progesterone via its nuclear PR receptors is essential for Rbbp7 expression in the stroma during the periimplantation period. There is previous evidence that the expression of RBBP7 could be upregulated by about 3 fold when MCF-7 cells were treated with estrogen for 24h, and RBBP7 can interact with ERα receptor to modulate estrogen responsiveness in a gene-specific manner [31, 45]. Therefore, the potential regulatory activity of estrogen on Rbbp7 expression, particularly in uterine luminal and glandular epithelium should not be ruled out.

Uterine decidualization in mice is characterized by extensive stromal cell proliferation and differentiation into decidual cells with polyploidy, which involves in dynamic cell cycle events [2]. Among various cell cycle related molecules including various cyclins, CDKs and cell cycle inhibitors, cyclin D3, a well-known regulator of mammalian cell proliferation [46], has been shown to be highly expressed in the decidualizing stromal cells within implantation chambers and essential for normal postimplantation uterine decidualization [47, 48]. Previous studies demonstrated that many key signaling and regulatory molecules such as heparin-binding EGF-like growth factor (HB-EGF) [49], interleukin 11-interleukin 11 receptor alpha (IL11-IL11Rα) [50, 51], Hox family transcription factor HOXA10 [52], Kruppel-like factor 9 (KLF9, previously known as BTEB1) [53], death effector domain-containing protein (DEDD) [54] were considered to regulate decidualization at least partially through cyclin D3. In this respect, we added a new line of evidence showing that Rbbp7 is critical for normal cyclin D3 expression in mESCs during decidualization. It is also interestingly to emphasize again, that Rbbp7 similar to Hoxa10 and Klf9 is a progesterone-responsive gene in stromal cells. However, it remained to explore whether these various key regulators function in parallel or in an up-down regulatory manner during stromal decidualization.

As a histone binding component of many histone modification complexes and chromatin remodeling complexes, Rbbp7 has been proposed to perform its function through affecting
complexes activity and regulating the substrate specificity [28, 55]. For example, Rbbp7 was reported to be essential for enzymatic activity of histone acetyltransferase Hat1, and its substrate recognition. When Rbbp7 was absent, the activity of Hat1 was very weak [56]. Consistent with previous notion that Rbbp7 mainly binds to H4 [19], and a preferential acetylation of H4 was detected by proteomics after intraluminal oil perfusion in mice [57], we observed a general decrease of acetylation level of H4 upon Rbbp7 silencing, while a hypoacetylation status of core histones is often correlated with gene repression [14]. There is evidence that induction of histone hyperacetylation by histone deacetylases inhibitor can upregulate both mRNA and protein expressions of cyclin D3 [58]; whereas depletion of K(lysine) acetyltransferase 2 (KAT2, also known as GCN5), a kind of histone acetyltransferase results in downregulation of cyclin D3 [59]. Since reduced cyclin D3 expression and impaired decidualization can also be observed in mESCs treated with histone acetyltransferases inhibitor C646, similar to those resulted from Rbbp7 knockdown, it is conceivable that Rbbp7 may perform its function via affecting histone acetyltransferases activity. Although Rbbp7 was reported to interact with Hat1 and modulate its activity mostly at acetylation sites of H4 [60], the function of this complex was reported to acetylate newly synthesized histones during the process of chromatin assembly and histone deposition [61]. Whether the complex could influence transcription has not been defined. Moreover, Rbbp7 can also interact with p300, another histone acetyltransferase, and facilitate p300-mediated transcription in Hela cells [62]. Therefore, it will be interestingly to further explore the respective interaction of Rbbp7 with differential histone acetyltransferase in future.

Nonetheless, we demonstrated in the present study that Rbbp7 is a potentially functional player in stromal cells essential for normal histone acetylation modification and cyclin D3 expression during postimplantation decidual development.

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**Figure legends**

**Figure 1** Rbbp7 is spatiotemporally expressed in the perimplantation uterus. A) *In situ* hybridization analysis of uterine *Rbbp7* with isotope labelled probe on days 1, 4, 5, 6 and 8 of pregnancy. Section of day 5 uteri hybridized with the sense probe served as negative control. Bar = 200 µm. B) Immunohistochemistry analysis of Rbbp7 on Days 1, 4, 5, 6 and 8 of pregnancy. The decidualized cells are indicated by arrows or arrowheads for respective large mononucleated or binucleated cells in B. Bar = 200 µm. e, embryo; bl, blastocyst; le, luminal epithelium; ge,
glandular epithelium; m, mesometrial pole; am, anti-mesometrial pole.

**Figure 2 Progesterone via nuclear PR receptor induces Rbbp7 expression in stromal cells.** A, B) Ovariectomized mice rested for one week and then were injected with 100ng E2 or 2mg P4 or 100ng E2 plus 2mg P4, the expression of Rbbp7 at mRNA level was detected at 1h, 2h and 24h (A) after injection, while protein level of Rbbp7 were investigated at 0h and 24h (B) after injection. The injection of oil served as control. Different letters represent statistical significance within each treatment (P<0.05). Bar = 50 µm. C) PR^-/- mice and control mice (PR^+/+) were ovariectomized, rested for one week, then were injected with 2mg P4, uteri were isolated at 0h and 24h after injection to detect the expression of Rbbp7 by IHC. Bar = 50 µm.

**Figure 3 Stromal Rbbp7 expressions in oil-induced decidualoma.** A) In situ hybridization analysis of Rbbp7 with digoxygenin labelled probe in both oil-infused side and control (con) side of uterus at 48h and 96h after oil infusion. Bar = 200 µm. B) Immunohistochemistry analysis of Rbbp7 in both oil-infused side and control side of uterus at 48h and 96h after oil infusion. The decidualized cells are indicated by arrows or arrowheads for respective large mononucleated or binucleated cells in B. Bar = 200 µm. le, luminal epithelium; ge, glandular epithelium; m, mesometrial pole; am, anti-mesometrial pole.

**Figure 4 Rbbp7 silencing via stealth RNA compromises uterine stromal cell proliferation and decidualization.** A) The location of Rbbp7 in stromal cells in vitro by immunofluorescence analysis. Rbbp7 was mainly located in nucleus. Bar = 75 µm. B-D) The knockdown efficiency of Rbbp7 in primary uterine stromal cells. B) The mRNA level of Rbbp7 decreased to lower than 20% in stealth RNA targeting Rbbp7 (siRNA)-transfected group compared with normal (nor) or control siRNA (con)-transfected group detected by real-time qPCR. Stromal cells were collected to extract RNA at 48h after transfection. The result represents mean ± SEM. ***P < 0.001. C) The protein level of Rbbp7 was reduced obviously 48h after transfected with Rbbp7 siRNA analyzed by western blotting using β-actin as loading control. D) The immunofluorescence analysis of Rbbp7 knockdown efficiency in stromal cells in vitro. Bar = 100 µm. E) Cell Proliferation Assay at indicated time points. The data are presented as mean ± SEM. *P<0.05. F) Immunofluorescence analysis of BrdU at 24h, 48h and 96h after BrdU incorporation. Bar = 100 µm. G) Statistical analysis of BrdU positive ratio by counting randomly selected images from 6 individual areas on the slides. The data are presented as mean ± SEM. *P<0.05. H) The detection of Dtprp mRNA level by RT-qPCR. The data represents mean ± SEM. *P<0.05. I) Alkaline phosphatase activity was detected in stromal cells by staining with BCIP/NBT Color Development Substrate. Bar = 200 µm. J) The relative alkaline phosphatase activity standardized to protein quality. Alkaline phosphatase activity was assessed at 405 nm, protein quality was assessed at 595nm. *P<0.05.

**Figure 5 Rbbp7 silencing attenuates uterine stromal cell cyclin D3 expression.** A) The co-localization of Rbbp7 and cyclin D3 in the implantation sites on day 6 and 8 of pregnancy. The
experiment was performed in two adjacent paraffin sections. Bar = 200 µm. e, embryo; le, luminal epithelium; m, mesometrial pole; am, anti-mesometrial pole. Arrows with same color represent the same cell having large mononuclear or two nuclei. B, C) The expression of cyclin D3 decreased in Rbbp7 siRNA-transfected group at both mRNA level (B) and protein level (C) during in vitro proliferation. The data represents mean ± SEM. ** P<0.01. *** P<0.001. D, E) Cyclin D3 was downregulated in Rbbp7 siRNA-transfected group at both mRNA level (D) and protein level (E) during in vitro decidualization. The data represents mean ± SEM. * P<0.05. ** P<0.01. *** P<0.001. con, control. (F) The immunofluorescence analysis of cyclin D3 at 96h after Rbbp7 knockdown in stromal cells. Cyclin D3 was reduced obviously. Scale bar, 100 µm.

**Figure 6 Rbbp7 silencing attenuates uterine stromal cell histone acetylation levels. A, B) The western blot analysis of common acetylation sites of H4 in control (con) and Rbbp7 knockdown groups (A) and the quantitative analysis by gray scanning of the bands (B), H4 was used as loading control. The data represents mean ± SEM. * P<0.05. C) The expression of Dtprp in stromal cells at 96h decreased in a dose-dependent manner when stromal cells were treated with histone acetyltransferases inhibitor C646. The data represents mean ± SEM. ** P<0.01. nor, normal. D) The protein level of acH4K5 in DMSO and C646 5 µM groups, H4 was used as loading control. The gray scanning analysis result is shown as mean ± SEM. ** P<0.01. E) The expression level of cyclin D3 decreased in C646 5 µM group compared with DMSO group detected by western blotting analysis. Protein lysates for western blot analysis were obtained at 48h after C646 treatment. The gray scanning analysis result is shown as mean ± SEM. *** P<0.001.
Figure 2
Figure 3
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Figure 6