Original Paper

High Mobility Group Box 1 Regulates Uterine Decidualization through Bone Morphogenetic Protein 2 and Plays a Role in Kruppel-Like Factor 5-Induced Stromal Differentiation

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Key Words

Hmgb1 • Klf5 • Bmp2 • Decidualization • Uterine stromal cell

Abstract

Background/Aims: High mobility group box 1 (Hmgb1) is associated with a variety of physiological processes including embryonic development, cell proliferation and differentiation, but little information is available regarding its biological role in decidualization. **Methods:** In situ hybridization, real-time PCR, RNA interference, gene overexpression and MTS assay were used to analyze the spatiotemporal expression of Hmgb1 in mouse uterus during the pre-implantation period, and explore its function and regulatory mechanisms during uterine decidualization. **Results:** Hmgb1 mRNA was obviously observed in uterine epithelium on day 2 and 3 of pregnancy, but its expression was scarcely detected on day 4 of pregnancy. With the onset of embryo implantation, abundant Hmgb1 expression was noted in the subluminal stromal cells around the implanting blastocyst at implantation sites. Meanwhile, the accumulation of Hmgb1 mRNA was visualized in the decidual cells. Hmgb1 advanced the proliferation of uterine stromal cells and induced the expression of prolactin family 8, subfamily a, member 2 (Prl8a2), a reliable differentiation marker for decidualization. In uterine stromal cells, cAMP analogue 8-Br-cAMP up-regulated the expression of Hmgb1, but the up-regulation was abrogated by protein kinase A (PKA) inhibitor H89. Silencing of Hmgb1 by specific siRNA impeded the induction of 8-Br-cAMP on Prl8a2. Further analysis evidenced that Hmgb1 was a critical mediator of Kruppel-like factor 5 (Klf5) function in stromal differentiation. Knockdown of bone morphogenetic protein 2 (Bmp2) prevented the up-regulation of Prl8a2 elicited by Hmgb1 overexpression, whereas addition of exogenous recombinant Bmp2 protein (rBmp2) reversed the repression of Hmgb1 siRNA on Prl8a2 expression. **Conclusion:** Hmgb1 may play an important role during mouse uterine decidualization.

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Published by S. Karger AG, Basel

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Introduction

Embryo implantation is the most critical step in the reproductive process and involves in an intricate discourse between the blastocyst and receptive uterus [1-3]. In mice, implantation takes place at the midnight of day 4 and becomes more prominent in the morning of day 5 of pregnancy [3, 4]. When the developed blastocyst attaches to uterine luminal epithelium, stromal cells proliferate and differentiate into decidual cells, a process known as decidualization which may accommodate embryonic growth and prevent uncontrolled trophoblast invasion [4-6]. It has been previously established that uterine-specific ablation of Kruppel-like factor 5 (Klf5), a zinc finger-containing transcription factor, resulted in female infertility due to defective implantation and decidualization [7], but its underlying mechanism remains poorly understood.

High mobility group box 1 (Hmgb1, also known as Hmg1) was the most abundant and well-studied member among Hmg family which was discovered as a group of non-histone nuclear proteins with high electrophoretic mobility and contained two DNA-binding HMG-box domains and an acidic C-terminal tail [8]. Hmgb1 was localized in the nucleus, where it might exert an important function for chromatin structure and further influence the transcription, replication, recombination, DNA repair and genomic stability [8, 9]. In addition to its nuclear role, Hmgb1 could also act as an extracellular signaling molecule to modulate the inflammation, immune response, autophagy and cancer [9-11]. Further analysis evidenced that Hmgb1 was highly expressed in mouse blastocyst and implicated in the regulation of preimplantation embryo development [12, 13]. Meanwhile, abundant Hmgb1 expression was also observed in human and rat uteri during pre-receptive phase, while receptive uteri exhibited a decline [14]. Administration of recombinant Hmgb1 into uterine horn of pregnancy rats led to pregnancy failure [14], suggesting a significant role of Hmgb1 in pregnancy events, but there is almost no data describing its physiological function in uterine decidualization.

Bone morphogenetic protein 2 (Bmp2) is a multifunctional growth factor belonging to the transforming growth factor β (TGF- β) superfamily and has been shown to be a key regulator of decidualization [4, 15]. Mice lacking Bmp2 in the uterus were sterile due to the inability to undergo decidualization [15]. However, little is known regarding the interplay of Bmp2 and Hmgb1 in stromal differentiation.

In this study, we found that Hmgb1 was visualized in the decidua and induced the proliferation and differentiation of uterine stromal cells through targeting Bmp2. Furthermore, Hmgb1 might mediate stromal cell differentiation elicited by cAMP and Klf5.

Materials and Methods

Animals

Mature mice (Kunming white strain) were caged in a controlled environment with a cycle of 14L:10D. All animal procedures were approved by the Institutional Animal Care and Use Committee of Jilin University. To confirm reproducibility of results, at least three mice per group were used in each stage or treatment in this study.

Pregnancy and pseudopregnancy

Adult female mice were mated with fertile or vasectomized males of the same strain to induce pregnancy or pseudopregnancy by co-caging, respectively (day 1=day of vaginal plug). On days 1-4, pregnancy was confirmed by recovering embryos from the oviducts or uterus. The implantation sites on day 5 were identified by intravenous injection of 0.1 ml of 1% Chicago blue (Sigma, St. Louis, MO) in 0.85% sodium chloride.

Delayed implantation and activation

To induce delayed implantation, pregnant mice were ovariectomized under ether anesthesia at 08:30–09:00h on day 4 of pregnancy. Progesterone (1 mg/mouse; Sigma) was injected subcutaneously to maintain delayed implantation from days 5 to 7. Estradiol-17 β (25 ng/mouse, Sigma) was given to progesterone-primed delayed-implantation mice to activate blastocyst implantation. The mice were sacrificed to collect uteri at 24 h after estrogen treatment. The implantation sites were identified by intravenous injection of Chicago blue solution. Delayed implantation was confirmed by flushing the blastocysts from the uterus.

Artificial induced decidualization

Artificial decidualization was induced by intraluminally infusing 25 μ l of sesame oil into one uterine horn on day 4 of pseudopregnancy, while the contralaterally uninjected horn served as a control. The mice were sacrificed to collect uteri at 48, 72 or 96 h after artificial induced decidualization. Decidualization was confirmed by weighing the uterine horn and histological examination of uterine sections.

In situ hybridization

Total RNAs from the mouse uteri were reverse-transcribed and amplified with Hmgb1 primers (5'-TTGAAGATATGGCAAAGGCT and 5'-GCATCAGGTTTCCTTTAGC). The amplified fragment (331 bp) of Hmgb1 was cloned into pGEM-T plasmid (pGEM-T Vector System, Promega, Madison, WI) and verified by sequencing. Hmgb1-containing plasmid was amplified with the primers for T7 and SP6 to prepare templates for labeling. Digoxigenin (DIG)-labeled antisense and sense cRNA probes were transcribed *in vitro* using a DIG RNA labeling kit (Roche Diagnostics GmbH, Mannheim, Germany).

Frozen sections (10 μ m) were mounted on 3-aminopropyltriethoxy-silane (Sigma)-coated slides and fixed in 4% paraformaldehyde solution in PBS. Hybridization was performed as described previously [16]. Sections were counterstained with 1% methyl green. The positive signal was visualized as a dark brown color. The sense probe was also hybridized and served as a negative control. There was no detectable signal from sense probes.

Real-time PCR

Total RNAs from cultured cells were isolated using TRIzol reagent (Roche) according to the manufacturer's instructions and reverse-transcribed into cDNA using MMLV reverse-transcriptase (Promega). For real-time PCR, cDNA was amplified using FS Universal SYBR Green Real Master (Roche) on LightCycler 96 Real Time Detection System. The conditions used for real-time PCR were as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were run in triplicate. The result was analyzed using LightCycler 96 Software. After analysis using the $2^{-\Delta\Delta C_t}$ method, data were normalized to Gapdh expression. Primer sequences were shown as follows: 5'-CCCGATGCTTCTGTCAACT and 5'-AACGAGCCTTGTCAGCCTTT (Hmgb1); 5'-CACGGATCTAGACATGCCC and 5'-ACGTCGTGGAACAGCAGAG (Klf5); 5'-CGCAGCTTCCATCACGAA and 5'-CACTGGGGACAGAACTTAAA (Bmp2). The other primer sequences used in this study were described previously [16].

Isolation of uterine stromal cells

Uterine stromal cells were isolated as previously described [16, 17]. Briefly, uterine horns from day 4 pregnant mice were split longitudinally and digested in HBSS containing 1% trypsin and 6 mg/ml dispase (Roche). The digested uteri were shaken lightly to remove sheets of luminal epithelial cells. The remaining tissues were washed three times with HBSS and incubated in HBSS containing 0.15 mg/ml collagenase I at 37°C for 30 min, followed by vigorously shaking until the supernatant became turbid. The supernatant were then passed through a 70 μ m wire gauze filter to eliminate epithelial sheets and centrifuged. The cell pellets were washed twice with HBSS and resuspended in complete medium consisting of DMEM-F12 (Sigma) with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen). The cells were inoculated to 6-well dishes at the concentration of 5×10^5 cells/well. After an initial culture for 30 min, the medium was changed to remove free-floating cells. The isolated stromal cells were further cultured in fresh complete medium at 37°C with 5% CO₂ before treatments or transfections.

RNA interference

The small-interfering RNA (siRNA) duplexes for targeting Hmgb1, Klf5 and Bmp2 as well as a scrambled sequence (control siRNA duplex, negative control) were designed and synthesized by GenePharma. The sequences were shown as follows: 5'-CAAGGCUCGUUAUGAAAGATT and 5'-UCUUUCAUAACGAGCCUUGTT (Hmgb1 siRNA 1); 5'-GCUUAUCCAUGGUGAUGUTT and 5'-ACAUCACCAUUGGAUAAGCTT (Hmgb1 siRNA 2); 5'-GGAUAUUGCUGCCUACAGATT and 5'-UCUGUAGGCAGCAUAUCCTT (Hmgb1 siRNA 3); 5'-AAGCUCACCUGAGGACUCAUU and 5'-UGAGUCCUCAGGUGAGCUUUU (Klf5 siRNA); 5'-GUUUCAGCACCGAAUUAATT and 5'-UUAUUUCGGUGCUGGAACTT (Bmp2 siRNA); 5'-UUCUCCGAACGUGUCACGUTT and 5'-ACGUGACACGUUCGGAGAATT (nonspecific scrambled siRNA, negative control). Transfections for siRNA were performed according to Lipofectamine 2000 protocol. Briefly, 100 pmol siRNA and 5 µl Lipofectamine 2000 were diluted in 250 µl Opti-MEM, respectively. After an incubation of 5 min, the diluted siRNA was mixed with diluted Lipofectamine 2000 and incubated for 20 min. The siRNA-Lipofectamine 2000 mixture was added onto 50-60% confluent uterine stromal cells for 6 h. Then the cells were cultured with complete culture medium and collected for analysis at 48 h.

Plasmid Construction and Transfection

Full-length Hmgb1 and Klf5 cDNA fragments were amplified by PCR from mouse uterus using the following primers: Hmgb1 (5'-GATATC (EcoR V) GAGGAAAATCAACTAAACAT and 5'-CTCGAG (Xho I) TTATTCATCATCATCATCTT) and Klf5 (5'-GAATTC (EcoR I) ATGCCACGCGGGTGCTGA and 5'-GATATC (EcoR V) CTCGCTCAGTTCTGGTGGC). The amplified products were purified and cloned into pGEM-T vector. The pGEM-T-Hmgb1, pGEM-T-Klf5 and pcDNA3.1 vector were cut by EcoR V/Xho I or EcoR I/EcoR V (TaKaRa, Dalian, China) at 37°C for 1 h, and then the fragments were ligated into pcDNA3.1 with T4 ligase (Promega) at 4°C overnight to construct pcDNA3.1-Hmgb1 and pcDNA3.1-Klf5, respectively. An empty pcDNA3.1 expression vector was served as control.

Transfection of uterine stromal cells was performed according to the manufacturer's protocol for Lipofectamine 2000. Briefly, 4 µg plasmid DNA was mixed with 250 µl Opti-MEM. The mixture was gently added into 250 µl Opti-MEM containing 7.5 µl Lipofectamine 2000. The solution was incubated for 20 min at room temperature and added onto 70-80% confluent uterine stromal cells in 1.5 ml of Opti-MEM medium. After transfection for 6 h, the cells were washed with PBS and then cultivated in complete culture medium. The cells were collected for analysis at 48 h.

Cell proliferation

Proliferation assays were performed using MTS reagent (Promega) according to the manufacturer's directions. Uterine stromal cells were seeded at a density of 1×10^5 /well in 96-well plates and cultured in the DMEM/F12 medium containing 2% heat-inactivated FBS. After transfection with Hmgb1 siRNA or overexpression plasmid, stromal cells were cultured for 48 h, at which time 20 µl of MTS reagent was added to each well and incubated for 4 h. Absorbance was measured at 490 nm using a 96-well plate reader. Every experiment was performed in triplicate.

Statistics

All the experiments were independently repeated at least three times. The significance of difference between two groups was compared by Independent-Samples T Test. The multiple comparisons were tested with one-way ANOVA. The differences were considered significant at $P < 0.05$. All statistical analysis was performed using SPSS19.0 software program (SPSS Inc., Chicago).

Results

Hmgb1 mRNA expression during early pregnancy

To explore the physiological role of Hmgb1 in early pregnancy, in situ hybridization was performed to localize its spatiotemporal expression in mouse uterus. The results exhibited that there was no detectable signal of Hmgb1 mRNA on day 1 of pregnancy (Fig. 1A). Hmgb1 mRNA was markedly presented in luminal and glandular epithelium on days 2 and 3 of pregnancy (Fig. 1B and C). On day 4 of pregnancy when uterus was the receptive

Fig. 1. In situ hybridization of Hmgb1 expression in mouse uteri during early pregnancy on days 1 (A), 2 (B), 3 (C), 4 (D), 5 (E, F), 6 (G), 7 (H) and 8 (I). 5-I, implantation site on day 5 of pregnancy; 5-N, inter-implantation site on day 5 of pregnancy. Asterisks indicate embryo. Bar=60µm.

status, Hmgb1 mRNA signal was scarcely detected (Fig. 1D). Accompanying with the onset of embryo implantation on day 5, Hmgb1 mRNA was highly expressed in the subluminal stromal cells around the implanting blastocyst, but undetected at the inter-implantation sites (Fig. 1E and F). From day 6 to 8 of pregnancy, a high level of Hmgb1 mRNA signal was noted in the decidua and embryos (Fig. 1G-I).

Hmgb1 mRNA expression during delayed implantation and activation

To determine whether Hmgb1 expression was dependent on the presence of an active blastocyst, delayed and activated implantation model was performed. The results showed that Hmgb1 mRNA signal was barely detected in the delayed uterus (Fig. 2A). After estrogen activated the dormant blastocyst to implant, Hmgb1 mRNA was obviously accumulated in the subluminal stroma surrounding the implanting embryo (Fig. 2B).

Hmgb1 mRNA expression under artificial decidualization

To further address the relevance between Hmgb1 and uterine decidualization, artificial decidualization model was employed. In the uninjected control uteri, Hmgb1 mRNA signal was scarcely found, whereas the decidualizing stromal cells displayed an abundant accumulation in the level of Hmgb1 mRNA at 48, 72 and 96 h after intraluminal oil infusion (Fig. 2C-H).

Effects of Hmgb1 on the proliferation and differentiation of uterine stromal cells

Since uterine decidualization was characterized by extensive proliferation and differentiation of stromal cells [4], we firstly investigated the effects of Hmgb1 on stromal cell proliferation. After three different Hmgb1 siRNAs were transfected

Fig. 2. In situ hybridization of Hmgb1 expression in mouse uteri. A and B, Hmgb1 expression during delayed implantation and activation. C-H, Hmgb1 expression under artificial decidualization. Delay, delayed implantation; Activation, activation of delayed implantation by estrogen; Control, uninjected uterine horn; Oil, oil-induced decidualization.

into uterine stromal cells, the expression of Hmgb1 mRNA were significantly down-regulated by Hmgb1 siRNA 1 and 2 compared with scrambled siRNA, but the former was more significant, while Hmgb1 expression was unaltered after treatment with Hmgb1 siRNA 3 (Fig. 3A). Therefore, Hmgb1 siRNA 1 was chosen for following experiments. Knockdown of Hmgb1 reduced the proliferation of uterine stromal cells (Fig. 3B). Conversely, transfection with Hmgb1 overexpression plasmid, which could distinctly enhance the expression of this mRNA, resulted in an increase in stromal cell proliferation (Fig. 3C and D). To further elucidate the molecular basis for the proliferative role of Hmgb1, we analyzed its effects on the expression of cyclin A1 (Ccna1), Ccnb1, Ccnb2, Ccnd1, Ccnd3 and Ccne1. The results showed that silencing of Hmgb1 by specific siRNA efficiently suppressed the expression of Ccnd3 in uterine stromal cells, whereas constitutive activation of Hmgb1 had the opposite effects (Fig. 3E and F). After transfection with Hmgb1 siRNA or overexpression plasmid, the expression of Ccna1, Ccnb1, Ccnb2, Ccnd1 and Ccne1 did not show any significant change (Fig. 3E and F).

To explore the role of Hmgb1 in stromal cell differentiation, we analyzed its effects on the expression of prolactin family 8, subfamily a, member 2 (Prl8a2) which is a reliable differentiation marker for decidualization [16, 17]. The results evidenced that after Hmgb1 siRNA treatment, the level of Prl8a2 mRNA exhibited an obvious reduction (Fig. 3G). On the contrary, when Hmgb1 overexpression plasmid was introduced into uterine stromal cells, the expression of Prl8a2 was significantly elevated (Fig. 3H).

Hmgb1 mediates the effects of cAMP on the differentiation of uterine stromal cells

It is generally considered that uterine decidualization is accompanied by elevated intracellular cAMP levels which prominently activates protein kinase A (PKA) signal transduction pathway [18, 19]. In uterine stromal cells, cAMP analogue 8-bromoadenosine-cAMP (8-Br-cAMP, 500 μ M) up-regulated the expression of Hmgb1 at 48 h, whereas PKA inhibitor H89 (10 μ M) abrogated the up-regulation of Hmgb1 elicited by 8-Br-cAMP (Fig. 4A). To delineate the role of Hmgb1 in cAMP-mediated stromal differentiation, we transfected stromal cells with Hmgb1 siRNA, then added 8-Br-cAMP and analyzed the expression of Prl8a2. The results showed that attenuation of Hmgb1 could hinder the induction of 8-Br-cAMP on Prl8a2 expression (Fig. 4B).

Hmgb1 mediates the effects of Klf5 on the differentiation of stromal cells

As is well known, Klf5 was indispensable for uterine decidualization [7]. In uterine stromal cells, siRNA-mediated down-regulation of Klf5 weakened the expression of stromal differentiation marker Prl8a2, while overexpression of Klf5 augmented the expression of Klf5 and Prl8a2 (Fig. 4C-E). Further analysis found that Hmgb1 expression was clearly diminished after treatment with Klf5 siRNA and strengthened after transfection with Klf5 overexpression plasmid (Fig. 4C and E). Neither suppression nor overexpression of Hmgb1 exhibited any statistically significant difference in the expression of Klf5 (Fig. 4F). We next investigated whether Hmgb1 might mediate the effects of Klf5 on the differentiation of stromal cells. After co-transfection with Klf5 overexpression plasmid and Hmgb1 siRNA, the expression of Prl8a2 in uterine stromal cells was distinctly restrained (Fig. 4G). In contrast, constitutive activation of Hmgb1 compensated the effects of Klf5 siRNA on Prl8a2 expression (Fig. 4H).

Hmgb1 regulates the differentiation of uterine stromal cells via Bmp2

Because Bmp2 was highly expressed in the decidua overlapping with the expression of Hmgb1 [20], we subsequently elucidated the relationship between Bmp2 and Hmgb1. Treatment with siRNA targeted to Bmp2, efficiently suppressed the level of Bmp2 mRNA, led to a prominent decline in the expression of Prl8a2, but had no obvious impact on the expression of Hmgb1 (Fig. 5A). Exogenous recombinant mouse Bmp2 protein (rBmp2, 100 ng/ml) raised the expression of Prl8a2, but did not change the expression of Hmgb1 (Fig. 5B). After transfection with Hmgb1 siRNA, Bmp2 expression was markedly attenuated, whereas

Fig. 3. Effects of Hmgb1 on the proliferation and differentiation of uterine stromal cells. A, Effects of Hmgb1 siRNAs on Hmgb1 mRNA expression in the uterine stromal cells. After transfection with control siRNA, Hmgb1 siRNA 1, siRNA 2 or siRNA 3, Hmgb1 mRNA expression was determined by real-time PCR. B, Effects of Hmgb1 siRNA on the proliferation of uterine stromal cells. After transfection with Hmgb1 siRNA, stromal cells were analyzed by MTS assay. C, Hmgb1 expression after uterine stromal cells were transfected with Hmgb1 overexpression plasmid. D, Effects of Hmgb1 overexpression on the proliferation of uterine stromal cells. E, Effects of Hmgb1 siRNA on the expression of Ccna1, Ccnb1, Ccnb2, Ccnd1, Ccnd3 and Ccne1. F, Effects of Hmgb1 overexpression on the expression of Ccna1, Ccnb1, Ccnb2, Ccnd1, Ccnd3 and Ccne1. G, Effects of Hmgb1 siRNA on Prl8a2 expression. H, Effects of Hmgb1 overexpression on Prl8a2 expression. Data are shown mean $\pm$ SEM. Asterisks denote significance ($P < 0.05$). NC, negative control; siHmgb1, Hmgb1 siRNA; Con, empty pcDNA3.1 vector; Hmgb1, Hmgb1 overexpression plasmid.

Fig. 4. Hmgb1 mediates the effects of cAMP and Klf5 on the differentiation of uterine stromal cells. A, Hmgb1 expression after uterine stromal cells were treated with 8-Br-cAMP, or both 8-Br-cAMP and H89. B, Hmgb1 mediated the effects of cAMP on Prl8a2 expression. After transfection with Hmgb1 siRNA and addition of 8-Br-cAMP, the expression of Prl8a2 was determined by real-time PCR. C, Effects of Klf5 siRNA on the expression of Klf5, Prl8a2 and Hmgb1. D, Klf5 expression after uterine stromal cells were transfected with Klf5 overexpression plasmid. E, Effects of Klf5 overexpression on the expression of Prl8a2 and Hmgb1. F, Effects of Hmgb1 siRNA or overexpression on the expression of Klf5. G, Hmgb1 siRNA blocked the effects of Klf5 overexpression on Prl8a2 expression. After co-transfection with Klf5 overexpression plasmid and Hmgb1 siRNA, the expression of Prl8a2 was determined by real-time PCR. H, Overexpression of Hmgb1 improved the effects of Klf5 siRNA on Prl8a2 expression. siKlf5, Klf5 siRNA; Klf5, Klf5 overexpression plasmid.

overexpression of Hmgb1 promoted the accumulation of Bmp2 mRNA in uterine stromal cells (Fig. 5C and D). Then we ascertained whether Bmp2 was involved in the Hmgb1-mediated stromal differentiation. Silencing of Bmp2 prevented the up-regulation of Prl8a2 elicited by Hmgb1 overexpression, whereas exogenous rBmp2 reversed the inhibitory effects of Hmgb1 siRNA on Prl8a2 expression (Fig. 5E and F).

Discussion

Hmgb1 is a multifunctional protein located inside and outside of the cell and has been shown to associate with a variety of physiological and pathological processes including embryonic development, cell proliferation and differentiation, inflammation, immunity, metabolism and cancer [8-11, 14, 21], but little information is available regarding its function in embryo implantation and decidualization. In this study, we showed that Hmgb1 mRNA was obviously observed in uterine epithelium on day 2 and 3 of pregnancy when uterus was pre-receptive, but its expression was scarcely detected with the attainment of uterine receptivity on day 4, which was consistent with that in human and rat uteri [14, 22, 23], suggesting a potential role of Hmgb1 in preparation for uterine receptivity which was required for successful embryo implantation. With implantation in progress on day 5, the accumulation of Hmgb1 mRNA was distinctly noted in the subluminal stroma surrounding the blastocyst at implantation sites, reinforcing the importance of Hmgb1 in embryo implantation.

It has been established that uterine decidualization is characterized by extensive proliferation and differentiation of stromal cells [4]. Previous studies have reported that Hmgb1 was implicated in regulating the proliferation of numerous cancer cells, embryonic and neural stem cells, mesangial cells, etc [8, 24-27]. The present evidence demonstrated that abundant Hmgb1 expression was seen in the decidua and induced the proliferation of uterine stromal cells. Further analysis displayed that Hmgb1 stimulated the expression of Ccnd3 which was a G1 phase cell cycle regulator for stromal cell proliferation [28], implying that Hmgb1 is involved in the transition from G1 into S phase. Indeed, knockdown of Hmgb1 in MGC-803 gastric cancer cells and embryonic stem cells resulted in an accumulation of cells in the G0/G1 phase along with the simultaneous reduction in the proportion of cells in the S phase, whereas administration of recombinant Hmgb1 protein to mesangial cells exhibited the opposite effects [25-27]. Simultaneously, Hmgb1 also promoted the expression of Prl8a2 which was a reliable differentiation marker for decidualization [16, 17], indicating a role of Hmgb1 in stromal differentiation. The notion was reinforced by the finding that silencing of Hmgb1 could impede the induction of Prl8a2 elicited by cAMP which facilitated the differentiation of uterine stromal cells accompanying with the elevated expression of Hmgb1. Taken together, these results indicate that Hmgb1 may play an important role during uterine decidualization.

Fig. 5. Hmgb1 regulates the differentiation of uterine stromal cells via Bmp2. A, Effects of Bmp2 siRNA on the expression of Bmp2, Prl8a2 and Hmgb1. B, Effects of rBmp2 on the expression of Prl8a2 and Hmgb1. C, Effects of Hmgb1 siRNA on Bmp2 expression. D, Effects of Hmgb1 overexpression on Bmp2 expression. E, Bmp2 siRNA weakened the effects of Hmgb1 overexpression on Prl8a2 expression. F, Exogenous rBmp2 reversed the effects of Hmgb1 siRNA on Prl8a2 expression. siBmp2, Bmp2 siRNA.

It is well-known that Klf5 is of great importance to uterine decidualization. Conditional deletion of uterine Klf5 resulted in compromised decidualization [7]. Meanwhile, there was a remarkably reduced response of the Klf5-deficient uteri to an artificial decidualogenic stimulus [7]. In uterine stromal cells, Klf5 might boost the expression of Hmgb1, implying that Hmgb1 may be a downstream target of Klf5 in the process of decidualization. This notion was supported by the present observation that knockdown of Hmgb1 could prevent the induction of Klf5 overexpression on Prl8a2, whereas constitutive activation of Hmgb1 improved the expression of Prl8a2 in the Klf5 siRNA-transfected stromal cells.

It has long been recognized that Hmgb1 was involved in transcriptional regulation of numerous genes [29]. In vascular smooth muscle cells, down-regulation of Hmgb1 could repress the expression of Bmp2 whose deficiency caused the inability of uterine stroma to undergo the decidual reaction to support further embryonic development [15, 30]. The similar result was also observed in uterine stromal cells. Moreover, knockdown of Bmp2 impeded the up-regulation of Prl8a2 elicited by Hmgb1 overexpression, whereas addition of rBmp2 reversed the repression of Hmgb1 siRNA on Prl8a2. Collectively, these results provide convincing evidence that Bmp2 is a critical mediator of Hmgb1 function in stromal differentiation.

Conclusion

Hmgb1 may regulate mouse uterine decidualization through targeting Bmp2 and plays a role in the differentiation of stromal cells elicited by cAMP and Klf5.

Acknowledgements

This work was financially supported by National Natural Science Foundation of China (31472158 and 31672503), and Graduate innovation Fund of Jilin University (2017085).

Disclosure Statement

The authors declare that they have no conflicts of interest.

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