Increased expression of HMGB1 in the implantation phase endometrium is related to recurrent implantation failure

Mi Han

Ruijin Hospital

Yi Cao

Fudan University Minhang Hospital

Wenjie Zhou

Ruijin Hospital

Mingjuan Zhou

Ruijin Hospital

Xiaowei Zhou

Ruijin Hospital

Dan Zhang

Ruijin Hospital

Bufang Xu

Ruijin Hospital

Aijun Zhang (✉ zhaj1268@163.com)

Ruijin Hospital <https://orcid.org/0000-0003-2879-9961>

Research Article

Keywords: recurrent implantation failure, high-mobility group box protein 1, cell adhesion, endometrial receptivity

Posted Date: July 24th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-695265/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Molecular Biology Reports on January 15th, 2022. See the published version at <https://doi.org/10.1007/s11033-021-06979-6>.

Abstract

Impaired endometrial receptivity is the main cause of recurrent implantation failure (RIF), however, its underlying mechanism is unclear. In this study, we found that HMGB1 expression was significantly decreased in the implantation phase endometrium in the control group (patients with tubal infertility who successfully achieved conception after the first embryo transfer) ($P = 0.006$). However, the expression levels of HMGB1 mRNA and protein were significantly upregulated during the implantation phase in endometrial tissues obtained from patients with RIF compared to those in the control group ($P = 0.001$), consistent with the results of genome-wide expression profiling. Moreover, *in vitro* assays showed that increased expression of HMGB1 in human endometrial epithelial cells cause marked deficiency in supporting blastocysts and human embryonic JAR cell adhesion, mimicking the process of embryo adhesion. However, overexpression of HMGB1 had no effect on cell proliferation and *in-vitro* decidualization in a human endometrial stromal cell line (T-HESCs) and in primary human endometrial stromal cells (HESCs). These findings indicate that increased HMGB1 levels suppressed the adhesion capability of epithelial cells, contributing to impaired endometrial receptivity in patients with recurrent implantation failure. This characteristic can be used as a target for detecting and treating recurrent implantation failure in clinical practice.

1. Introduction

Recurrent implantation failure (RIF), defined as the absence of clinical pregnancy following more than three *in vitro* fertilization-embryo transfer (IVF-ET) or intracytoplasmic sperm injection (ICSI) treatments with a total of at least four good-quality embryos (score ≥ 7 or grade of 3BB or better) [1], accounts for approximately 10% of cases of sterility in women during IVF-ET treatment [2]. RIF is not only a challenge for clinicians but also increases the economic burden and psychological trauma for patients [3]. RIF etiologies are complex and include maternal endocrine abnormality, hydrosalpinx, intrauterine adhesions, etc. [4–6]. However, the precise mechanisms of RIF are not well-understood. Endometrial receptivity and embryo quality are the main factors influencing the embryo implantation rate; however, it was reported that impaired endometrial receptivity is the major cause of RIF [7].

Endometrial receptivity refers to the ability of the endometrium to allow embryos to adhere, invade, and induce a series of changes in endometrial cells, and finally accept embryo implantation [8]. Factors that may affect endometrial receptivity include uterine fibroids [9], intrauterine adhesions [10], endometriosis [11], and hydrosalpinx [12], among others. Establishment of stable adhesion between the embryo and maternal tissue is crucial for endometrial receptivity acquisition [13]. Many adhesion molecules have been identified, such as the endometrial receptivity marker ITGB3, which interacts with osteopontin to participate in adhesion of the embryo to the epithelium [14]. Nevertheless, the mechanism of impaired endometrial receptivity is not well-understood. Moreover, few effective treatments have been developed for patients with infertility showing low endometrial receptivity in clinical practice [15].

High-mobility group box protein 1 (HMGB1) is a non-histone nucleoprotein abundantly expressed in most mammalian cells [16] and participates in several cellular processes. For example, increased HMGB1 expression in astrocytes reduced tumor cell adhesion [17]. Furthermore, some researchers showed that HMGB1 expression was significantly increased in unexplained recurrent spontaneous abortion [18]. In rats, exogenous administration of recombinant human HMGB1 protein led to pregnancy failure, indicating a crucial role for HMGB1 in embryo implantation [19]. Our previous genome-wide expression profiling analysis showed that HMGB1 expression was significantly increased in the implantation phase endometrium in patients with RIF compared to in the control group [20]. However, the role of HMGB1 in RIF remains unclear. In this study, we examined the effect of high HMGB1 expression on endometrial receptivity in RIF.

2. Materials And Methods

2.1 Clinical sample collection

Uterine tissues were collected via pipe suction curettage (Wallace) from patients who underwent IVF-ET/ICSI treatment at the Reproductive Medical Center of Ruijin Hospital, School of Medicine, Shanghai Jiao Tong University between January 2016 and December 2020. All patients signed an informed consent form before they were recruited in the study. Patients with a history of tubal infertility who successfully achieved conception after the first embryo transfer were recruited as the control group ($n = 38$) and divided to three phases of the menstrual cycle: early proliferative phase (days 4–5, $n = 10$); late proliferative phase (days 11–13, $n = 10$); implantation phase (days 20–23, $n = 18$). Patients with no pregnancy after at least three embryo transfers including a total of ≥ 4 good-quality embryos were recruited as the RIF group ($n = 18$). The inclusion criteria for all participants included: 25–35 years old, regular ovulatory cycles with every 28–32 days, normal serum levels including follicle stimulating hormone ($\text{FSH} < 10 \text{ mIU/mL}$), luteinizing hormone ($\text{LH} < 10 \text{ mIU/mL}$) and estradiol ($\text{E}_2 < 50 \text{ mIU/mL}$) on day 3 of the menstrual cycle. The exclusion criteria for all patients included: presence of intrauterine pathology, hydrosalpinx, salpingitis, polycystic ovary syndrome, endometriosis, adenomyosis, chromosome abnormalities, or autoimmune disease, and any intrauterine device or contraceptive drug within the last 6 months.

For endometrial biopsy, specimens collected from the control group in the three phases were used to analyze differences in the HMGB1 phase-specific expression in the normal natural menstrual cycle (Supplementary Table 1). The implantation phase endometrium from 18 patients in the control group and 18 patients in the RIF group was collected on day $\text{LH} + 7$ to examine the expression of HMGB1 and HOXA10 (Supplementary Table 2). As we described previously [21], patients were monitored for follicle development by transvaginal ultrasound (Hitachi EUB-2000; Tokyo, Japan) until the diameter of the dominant follicle was $\geq 15 \text{ mm}$, and then serum LH and E2 levels were quantified daily. The day on which the LH level peaked ($\geq 20 \text{ mIU/mL}$) was considered as day LH 0; the window/implantation phase was

defined as day LH + 7. To isolate primary endometrial cells, the endometrium was collected from the control group (late proliferative phase, n = 12).

2.2 Microarray assay

Whole-genome gene expression profiling was conducted using uterine tissues from three patients with RIF and three tissues from the control group in the implantation phase as described previously [21]. Data were analyzed using the Gene Expression Omnibus database (GSE103465), and genes were considered as differentially expressed when they showed a fold-change ≥ 2 and adjusted P -value ≤ 0.05 .

2.3 Real-time reverse transcription PCR (qRT-PCR)

Total RNA was extracted from cultured cells and endometrial biopsy tissues using the TaKaRa9767 kit in accordance with the manufacturer's instructions (Takara, Shiga, Japan). Next, 1 μ g RNA was reverse-transcribed into cDNA using Primescript Reverse transcriptase (Takara). The synthesized template cDNA, gene-specific primers (Supplementary Table 3), and fluorescent probes (Takara) were used for qRT-PCR. PCR was conducted on a QuantStudio™ 6 Flex Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The mRNA expression level was normalized to that of GAPDH. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method [22].

2.4 Western blotting analysis

Total protein was extracted from endometrial biopsy tissues and cultured cells using RIPA buffer (Thermo Fisher Scientific) supplemented with 1% protease inhibitor cocktail (Roche, Basel, Switzerland). After centrifuging the samples at 12,000 $\times g$ for 30 min at 4°C, the protein-containing supernatants were collected and with 30 μ g protein per sample separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were blocked and then incubated with primary antibodies against HOXA10 (ab191470, Abcam, Cambridge, UK; 1:1,000), HMGB1 (ab79823, Abcam; 1:10,000) and GAPDH (5174, Cell Signaling Technology, Danvers, MA, USA; 1:500) for 12 h at 4°C. After washing the membranes four times with PBS, they were incubated with secondary antibodies. Specific protein bands were visualized using an Odyssey Infrared Imager (LI-COR, Lincoln, NE, USA).

2.5 Immunohistochemical staining

Immunohistochemical (IHC) staining was performed on 5- μ m sections from 4% paraformaldehyde-fixed paraffin-embedded endometrial tissue samples. The slides were incubated with 5% bovine serum albumin (Amresco, Solon, OH, USA) (25°C, 30 min), and then incubated with anti-HMGB1 (dilution 1:400, ab79823, Abcam) and normal rabbit IgG antibodies overnight at 4°C. Images were acquired using an Olympus BX53M fluorescence microscope (Tokyo, Japan). The H-score was calculated to determine the expression of HMGB1 based on the percentage of cells stained at each intensity level, ranging from 3 to 0 (equaling to strong, moderate, weak, and negative staining, respectively).

2.6 Cell culture and transfection

Isolation of primary human endometrial stromal cells (HESCs) and primary human endometrial epithelial cells (HEECs) from endometrial samples was performed as described previously [20]. Briefly, fresh endometrial biopsy samples were rinsed twice with PBS, and then minced and digested with 1 mg/mL collagenase type IV (Thermo Fisher Scientific) at 37°C for 30–45 min. After enzymatic digestion, the mixture was passed through a 100-mm sieve followed by a 40-mm sieve, with HESCs collected by passing the mixture through a 40-µm sieve and HEECs collected on the opposite side of the 40-µm sieve. Ishikawa cells (human endometrial epithelial cell line) and JAR cells (human trophoblastic cell line) were obtained from the European Collection of Authenticated Cell Cultures (Salsbury, UK). The human endometrial stromal cell line (T-HESCs) were obtained from American Type Culture Collection (Manassas, VA, USA). All cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum at 37°C.

Negative control and HMGB1 over-expressing plasmids were purchased from GeneCopoeia (Rockville, MD, USA) and transfected into cells using transfection reagent (Roche) following the manufacturer's instructions.

2.7 Proliferation assay

The capacity for cell proliferation was examined using Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) in endometrial stromal cells (ESCs) including T-HESCs and HESCs. After 2 days of plasmid transfection, the cells were seeded into 96-well plates at 2000 cells/well and 10 µL of CCK-8 reagent was added to each well. After incubating at 37°C for 2 h, the optical densities were determined at 450 nm excitation using a microplate reader.

2.8 Cell migration assay

Ishikawa cells and HEECs were transfected with negative control and HMGB1 over-expressing plasmids and plated into 6-well plates. After transduction for 3 days when the cell confluence reached 80–90%, the cells were cultured in DMEM containing 0.1% fetal bovine serum for 24 h to achieve deprivation conditions. A vertical linear scratch was created in the Ishikawa monolayer cells using a 200-µL micropipette tip, with the floating cells removed by washing with sterile PBS. The cell migration distance was measured under a light microscope via Image J 1.46r (Nunes and Dias, NIH, Bethesda, MD, USA) at 0, 24, and 48 h after continuous culturing of the scratched cells in serum deprivation media at 37°C.

2.9 Embryo adhesion assay

The co-culture model of “embryo” adhesion was constructed as described by Jemma Evans et al. (2020) [23]. Briefly, HEECs were isolated from four late proliferative-phase endometrium samples from controls as described above. Ishikawa cells, possessing apical adhesiveness and similar characteristics of endometrial luminal and glandular epithelium, were commonly used to study endometrial receptivity and embryo adhesion [24–26]. Ishikawa cells and HEECs transfected with HMGB1 over-expressing or negative control plasmids were grown to confluence in a 48-well plate. Endometrial cells were then treated with a hormonal paradigm to mimic hormonal exposure throughout the menstrual cycle: cells

were primed with 10^{-8} M β -estradiol (E2758, Thermo Fisher Scientific, referred to as E) for 24 h, and then treated with combined E and 10^{-7} progesterone (V900699, Thermo Fisher Scientific, referred to as P) for 24 h, followed by treatment with E/P and 10 IU human chorionic gonadotropin for 24 h. An adhesion assay was then performed. A total of 44 hatched blastocysts from C57BL/6 mice with a normal morphology was cocultured with confluent monolayers of acceptable HEECs for 6 h in DMEM/F12 complete medium. After washing the cells three times with PBS, images of the adherent blastocysts were captured for morphological examination with a TS2 microscope (Nikon, Tokyo, Japan). JAR cells (human trophoblastic cell line) labeled with PKH26 (PKH26GL-1KT, Abcam) for 10 min at 37°C were then added to fully confluent endometrial epithelial cells (EECs) including Ishikawa cells and HEECs monolayers for 1 h at 37°C, followed by vigorous agitation three times at 140 rpm for 5 min before refreshing the medium. The attached JAR cells were then imaged with a fluorescence microscope (Leica V3.8, Wetzlar, Germany).

2.10 In vitro decidualization experiment

HESCs and T-HESCs were used for decidualization experiments. After 3 days of plasmid transfection, HESCs and T-HESCs were cultured in serum-free DMEM containing estradiol (10 nM, E2758, Sigma, St. Louis, MO, USA), progesterone (1 μ M V900699, Sigma), and 8-Br-cAMP (1 mM, ab141448, Abcam) for 3 and 8 days, respectively. Total RNA was extracted to analyze the expression of decidual markers: prolactin (PRL) and insulin-like growth factor-binding protein 1 (IGFBP1).

2.11 Statistical analysis

All experiments were independently performed at least three times. The results are shown as the mean $\pm$ standard error of the mean (SEM) and analyzed using SPSS software (version 23.0, SPSS, Inc., Chicago, IL, USA). Data were analyzed using Student's *t*-test between two groups and one-way analysis of variance among more than two groups. Data were considered as statistically significant when $P < 0.05$.

3. Results

3.1 Baseline characteristics

The characteristics of the control and RIF groups are shown in Supplementary Table 2. There were no significant differences between groups in average age, body mass index (BMI), follicle-stimulating hormone (FSH), luteinizing hormone (LH), or estradiol (E_2), although the number of embryo transfers differed significantly ($P < 0.001$). Similarly, there were no significant differences among the different phases of the menstrual cycles, as indicated by demographic characteristics (Supplementary Table 1).

3.2 HMGB1 expression in the endometrium of the menstrual cycles

In the control group endometrium, the mRNA levels of HMGB1 were high during the proliferative phases and significantly decreased during the implantation phase (Fig. 1a). However, HMGB1 protein levels

increased gradually from the early to late proliferative phase, and then decreased during the implantation phase (Fig. 1b,c). IHC staining showed that HMGB1 was localized in the stroma and epithelium of the human endometrium, and mostly located in the epithelium (Fig. 1d–f).

3.3 HMGB1 expression significantly increased in the RIF group during the implantation phase

According to our previous microarray results [20], the HMGB1 expression level was increased in the RIF group (fold-change = 2.00, $P < 0.05$, Fig. 2a). Consistently, qRT-PCR showed that the mRNA levels of HMGB1 were significantly increased in the RIF group during the implantation phase ($P < 0.01$) (Fig. 2b). Similarly, as shown in Fig. 2c and 2d, HMGB1 protein levels were considerably increased in the endometrium of patients with RIF during the implantation phase ($P < 0.001$), and HOXA10 protein expression was significantly lower in the RIF group. IHC staining showed that HMGB1 expression in the implantation phase endometrium of the RIF group was increased compared to in the control group (Fig. 2e,f), in line with the above-mentioned findings. Through receiver operating characteristic curve analysis, we verified the potential functional role of HMGB1 in endometrial receptivity, as the area under the curve was 0.8878 [0.803–0.972] for tissue HMGB1 measurements (Fig. 2g).

3.4 HMGB1 overexpression did not influence cell proliferation and decidualization of T-HESCs and HESCs

Based on the higher expression of HMGB1 in the endometrial stroma of RIF compared to in controls, HMGB1-overexpressing T-HESCs and HESCs were successfully constructed to examine the impact of HMGB1 on biological function, including stromal cell proliferation and *in vitro* decidualization. As shown in Fig. 3, HMGB1 overexpression did not affect cell proliferation or decidualization in T-HESCs and HESCs.

3.5 HMGB1 overexpression in EECs inhibited embryo adhesion in vitro

HMGB1-overexpressing HEECs and Ishikawa cells were successfully constructed, as confirmed by qRT-PCR and western blotting. Elevated HMGB1 levels had no effect on the migration ability of EECs (Fig. 4). However, overexpression of HMGB1 suppressed cell adhesion of embryonic JAR cells onto Ishikawa cells (Fig. 5a) and HEECs (Fig. 5c). The fluorescence intensity of fluorescence-labeled JAR cells adherent on over-expressing HMGB1 EECs cells was decreased compared to that on control EECs (Fig. 5b,d). We further examined a heterologous co-culture model of “embryo” adhesion. As shown in Fig. 5e, 44 mouse embryos were randomly assigned to the two groups in five parallel experiments, after co-culture for 6 h with the acceptable HEECs, in the negative control group, out of 22 blastocysts, 13 attached; however, in the HMGB1-overexpression group, of 22 blastocysts, 5 attached. HMGB1-overexpression in EECs significantly inhibited embryo attachment to the endometrial epithelium (Fig. 5f,g).

4. Discussion

Reproductive failure is a common social and economic issue delaying parenthood [27]. Although remarkable advances have been made in IVF-ET in clinical pregnancy, the technique fails repeatedly for several couples who are infertile [28]. Currently, RIF, largely defined as implantation failure due to impaired endometrial receptivity, is a challenging clinical problem in assisted reproductive technology [7]. Therefore, it is imperative to determine the potential mechanism of RIF. In this study, we found that HMGB1 overexpression impaired endometrial receptivity by affecting cell adhesion between the embryo and endometrial epithelium.

We previously identified CDYL [29] and PIBF1 [20], which were consistent with HOXA10 [21], an accepted endometrial receptivity marker, as expressed dynamically in the endometrium of the normal menstrual cycles in the control group. The dynamic expression changes of the mentioned above molecules in the different phases of normal menstrual cycles suggested that they play important roles in the acquisition of endometrial receptivity. Similarly, HMGB1 expression showed dynamic changes during the menstrual cycles. During normal menstrual cycles in control group, HMGB1 expression in the endometrium gradually increased from the early to late proliferative phase but decreased during the implantation phase, indicating that decreased expression of HMGB1 during the implantation phase is favorable for the acquisition of endometrial receptivity, in other words, increased expression of HMGB1 may inhibit embryo implantation.

In our previous study, genome-wide expression profiling suggested that HMGB1 expression was significantly increased in patients with RIF during the implantation phase, which was verified by qRT-PCR and western blotting in this study. Furthermore, the endometrial receptivity marker HOXA10 was significantly decreased in the RIF group, supporting impaired endometrial receptivity in patients with RIF. In addition, receiver operating characteristic analysis revealed the high sensitivity and specificity of HMGB1 in endometrial tissues for discriminating endometrial receptivity. Additionally low expression of HMGB1 in the implantation phase may be necessary for establishing endometrial receptivity, and increased expression of HMGB1 may contribute to impaired endometrial receptivity in patients with RIF.

IHC staining was performed to identify HMGB1 localization in the endometrium. HMGB1 was localized in the stroma and epithelium in the human endometrium. The proliferation and decidualization capability of ESCs, in addition to the migration and adhesion capability of EECs, were essential for the establishment of endometrial receptivity [20, 30]. To examine the mechanism by which HMGB1 affects endometrial receptivity, we constructed HMGB1-overexpressing ESCs and EECs to evaluate changes in cell biology processes in the negative control group. *In vitro* experiments with plasmid transfection to overexpress HMGB1 in T-HESCs and HESCs showed no effect on IGFBP1 and PRL (decidualization markers) levels, indicating that increased HMGB1 levels did not affect endometrial decidualization. However, Fig. 5 showed that overexpression of HMGB1 significantly inhibited the adhesion of EECs to JAR cells. Additionally, in the heterologous co-culture model of “embryo” adhesion, HMGB1 overexpressing in EECs displayed a marked deficiency in supporting blastocyst adhesion, which mimic the process of embryo

adhesion. It was also reported that high expression of HMGB1 decreased adhesion function of tumors cells [14]. Particularly, adhesion between the embryo and maternal tissue is well-known as a vital step in successful implantation [20, 31]. These findings suggest that overexpression of HMGB1 inhibited embryo adhesion, likely impacting endometrial receptivity. Future research should focus on the underlying mechanisms by which increased HMGB1 expression decreases endometrial receptivity in patients with RIF.

Conclusion

This study demonstrated that HMGB1 expression showed dynamic changes during normal menstrual cycles and decreased during the implantation phase. However, the HMGB1 expression level significantly increased in the endometrium in patients with RIF. Moreover, increased expression of HMGB1 suppressed the adhesion capability of epithelial cells. These results suggest that increased HMGB1 expression is harmful for endometrial receptivity and consequently leads to RIF, and may be useful for predicting endometrial receptivity.

Declarations

Acknowledgements: The authors thank all of the professors at the Institute of State Key Laboratory of Medical Genomics, Shanghai Ruijin Hospital for their help.

Funding: This work was supported by the National Natural Science Foundation of China [grant numbers 82071596 and 81701513].

Conflicts of Interest: The authors declare that no conflicts of interest exist

Availability of data and material: Not applicable

Authors' contributions: MH: protocol/project development, data collection or management, data analysis, manuscript writing/editing; YC: data collection or management, data analysis; WZ: conceptualization; MZ: data curation; XZ: data curation; DZ: funding acquisition, resources; BX: writing - review and editing, resources; AZ: writing - review and editing, funding acquisition.

Ethics approval: The study has been granted by the Institutional Ethics Committee of Ruijin Hospital, School of Medicine, Shanghai Jiao Tong University (2012-57). All patients signed an informed consent form before they were recruited in the study.

Consent to participate: Consent to participate was obtained from all individuals included in the study.

Consent to publish: Consent to publish relevant data was taken from all participants.

References

1. Xia L, Zhao S, Xu H, Wu X, Zhang A, Niu Z (2020) Miscarriage Rate Is High With Frozen-Thawed Blastocysts Arising From Poor-Quality Cleavage Stage Embryos. *Front Endocrinol (Lausanne)* 11:561085
2. Cimadomo D, Craciunas L, Vermeulen N et al (2021) Definition, diagnostic and therapeutic options in recurrent implantation failure: an international survey of clinicians and embryologists. *Hum Reprod* 36:305–317
3. Koot YEM, Hviid Saxtorph M, Goddijn M et al (2019) What is the prognosis for a live birth after unexplained recurrent implantation failure following IVF/ICSI? *Hum Reprod* 34:2044–2052
4. Cakiroglu Y, Tiras B (2020) Determining diagnostic criteria and cause of recurrent implantation failure. *Curr Opin Obstet Gynecol* 32:198–204
5. Sheikhansari G, Pourmoghadam Z, Danaii S et al (2020) Etiology and management of recurrent implantation failure: A focus on intra-uterine PBMC-therapy for RIF. *J Reprod Immunol* 139:103121
6. Bashiri A, Halper KI, Orvieto R (2018) Recurrent implantation failure-update overview on etiology, diagnosis, treatment and future directions. *Reprod Biol Endocrinol* 16:121
7. Craciunas L, Gallos I, Chu J, Bourne T et al (2019) Conventional and modern markers of endometrial receptivity: a systematic review and meta-analysis. *Hum Reprod Update* 25:202–223
8. Lessey BA, Young SL (2019) What exactly is endometrial receptivity? *Fertil Steril* 111:611–617
9. Pirtea P, Cicinelli E, De Nola R et al (2021) Endometrial causes of recurrent pregnancy losses: endometriosis, adenomyosis, and chronic endometritis. *Fertil Steril* 115:546–560
10. Zhang H, Zhang Q, Zhang J et al (2021) Urinary bladder matrix scaffolds improve endometrial regeneration in a rat model of intrauterine adhesions. *Biomater Sci* 8:988–996
11. Ziegler D, Frydman R (2021) Recurrent pregnancy losses, a lasting cause of infertility. *Fertil Steril* 115:531–532
12. Kong C, Sun L, Zhang M et al (2016) miR-133b Reverses the Hydrosalpinx-induced Impairment of Embryo Attachment Through Down-regulation of SGK1. *J Clin Endocrinol Metab* 101:1478–1489
13. Achache H, Revel A (2006) Endometrial receptivity markers, the journey to successful embryo implantation. *Hum Reprod Update* 12:731–746
14. Aplin JD, Ruane PT (2017) Embryo-epithelium interactions during implantation at a glance. *J Cell Sci* 130:15–22
15. Hernandez-Vargas P, Munoz M, Dominguez F et al (2020) Identifying biomarkers for predicting successful embryo implantation: applying single to multi-OMICs to improve reproductive outcomes. *Hum Reprod Update* 26:264–301
16. Tang Y, Zhao X, Antoine D et al (2016) Regulation of posttranslational modifications of HMGB1 during immune responses. *Antioxid Redox Signal* 24:620–634
17. Wu R, Yan Y, Ma C et al (2019) HMGB1 contributes to SASH1 methylation to attenuate astrocyte adhesion. *Cell Death Dis* 10:417

18. Zou H, Yin J, Zhang Z et al (2020) Destruction in maternal-fetal interface of URSA patients via the increase of the HMGB1-RAGE/TLR2/TLR4-NF-kappaB signaling pathway. *Life Sci* 250:117543
19. Bhutada S, Basak T, Savardekar L et al (2014) High mobility group box 1 (HMGB1) protein in human uterine fluid and its relevance in implantation. *Hum Reprod* 29:763–780
20. Zhou M, Xu H, Zang D et al (2020) Decreased PIBF1/IL6/p-STAT3 during the mid-secretory phase inhibits human endometrial stromal cell proliferation and decidualization. *J Adv Res* 30:15–25
21. Guo F, Si C, Zhou M et al (2018) Decreased PECAM1-mediated TGF-beta1 expression in the mid-secretory endometrium in women with recurrent implantation failure. *Hum Reprod* 33:832–843
22. Li D, Chen Q, Wei G et al (2020) Hypoxia-Induced miR-137 Inhibition Increased Glioblastoma Multiforme Growth and Chemoresistance Through LRP6. *Front Oncol* 10:611699
23. Evans J, Walker KJ, Bilandzic M et al (2020) A novel "embryo-endometrial" adhesion model can potentially predict "receptive" or "non-receptive" endometrium. *J Assist Reprod Genet* 37:5–16
24. Ling c, Zhou S, Dimitriadis W E (2020) Chloride intracellular channel 4 is dysregulated in endometrium of women with infertility and alters receptivity. *Biochem Biophys Res Commun* 531:490–496
25. Hannan N, Paiva P, Dimitriadis E et al (2010) Salamonsen, Models for study of human embryo implantation: choice of cell lines? *Biol Reprod* 82:235–245
26. Zhou W, Santos L, Dimitriadis E (2020) Characterization of the role for cadherin 6 in the regulation of human endometrial receptivity. *Reprod Biol Endocrinol* 18:66
27. Nowak I, Wilczynska K, Wilczynski JR et al (2017) KIR, LILRB and their ligands' genes as potential biomarkers in recurrent implantation failure. *Arch Immunol Ther Exp (Warsz)* 65:391–399
28. Kushnir VA, Barad DH, Albertini DF et al (2017) Systematic review of worldwide trends in assisted reproductive technology 2004–2013. *Reprod Biol Endocrinol* 15:6
29. Zhou X, Xu B, Zhang D et al (2020) Loss of CDYL Results in Suppression of CTNNB1 and Decreased Endometrial Receptivity. *Front Cell Dev Biol* 8:105
30. Cha J, Sun X, Dey SK (2012) Mechanisms of implantation: strategies for successful pregnancy. *Nat Med* 18:1754–1767
31. Yu M, Wang J, Liu S et al (2017) Novel function of pregnancy-associated plasma protein A: promotes endometrium receptivity by up-regulating N-fucosylation. *Sci Rep* 7:5315

Figures

Figure 1

HMGB1 expression decreased in the implantation phase endometrium in the control group. (a) QRT-PCR, (b) western blotting, and (c) quantification analysis of HMGB1 during different menstrual cycles (n = 10 per group). (d) Representative images of HMGB1 in different menstrual phase endometrial tissues analyzed via IHC staining (n = 5 per group). (e and f) Quantification analysis of HMGB1 protein in endometrial epithelium and stroma in different menstrual phase endometrium, the bottom row contains magnified pictures of the portion of the original image indicated by the red square. The scale bar represents 50 μ m. MS, implantation phase; EP, early proliferative phase; LP, late proliferative phase. Data are presented as the mean $\pm$ SEM. *P < 0.05; ***P < 0.001

Figure 2

HMGB1 was significantly increased in the implantation phase endometrial tissues with RIF. (a) HMGB1 expression in the control and RIF groups according to the microarray assay results ($n = 3$ per group). (b) qRT-PCR, (c) western blotting, and (d) quantification analysis of HOXA10 and HMGB1 between the control and RIF groups ($n = 18$ per group). (e) Representative pictures of HMGB1 between the control and RIF groups analyzed by IHC staining ($n = 5$ per group), and (f) quantification analysis of HMGB1 expression in endometrial stroma and epithelium of the control and RIF groups, the bottom row shows magnified images of the portion of the original image indicated by the red square. The scale bar represents 50 μ m. (g) ROC curve plotting of the sensitivity and specificity and optimal cutoff value for endometrium for HMGB1 measurements. Data are presented as the mean $\pm$ SEM. ** $P < 0.01$

Figure 3

Changes in stromal cell proliferation and decidualization in HMGB1-overexpressing ESCs. (a and c) Cell proliferation after HMGB1 overexpression in T-HESCs and HESCs using CCK-8. (b and d) Expression levels of decidual markers including PRL and IGFBP1 in T-HESCs and HESCs after HMGB1 overexpression during in vitro decidualization. Data are presented as the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$

Figure 4

HMGB1 overexpression in the EECs caused no significant changes in cell migration. (a and b) Effects of HMGB1 on cell migration in control and HMGB1-overexpression Ishikawa cells by wound-healing assay (c and d) Wound healing assay with control and HMGB1-overexpression HEECs cells. Ishikawa cells and HEECs were transfected with the control or HMGB1-overexpressing plasmid. Data are presented as the mean $\pm$ SEM

Figure 5

HMGB1 overexpression in the EECs inhibited embryo adhesion. (a and c) Representative images showing fluorescence-labeled JAR cells adherent on co-cultured over-expressing HMGB1 Ishikawa cells and HEECs. (b and d) Quantification of the adherent fluorescence intensity of fluorescence-labeled JAR cells. (e) Statistics of the number of co-cultured and finally adhered blastocysts in the NC group and HMGB1-OE groups. NC, negative control; HMGB1-OE, HMGB1-overexpression. (f and g) Representative images of adhesive mouse blastocysts (red arrow) and blastocyst adhesion rate between the two groups. Data are presented as the mean $\pm$ SEM. ** $P < 0.01$; *** $P < 0.001$

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTable1.docx](#)
- [SupplementaryTable2.docx](#)
- [SupplementaryTable3.docx](#)