Decreased PIBF1/IL6/p-STAT3 during the mid-secretory phase inhibits human endometrial stromal cell proliferation and decidualization

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\textbf{Highlights}

\begin{itemize}
  \item PIBF1 levels peaked in the mid-secretory phase of endometrium.
  \item PIBF1 expression decreased in the mid-secretory endometrium of RIF patients.
  \item PIBF1 regulated HESC proliferation and decidualization via IL6/p-STAT3 signaling.
  \item The IL6/p-STAT3, Ki-67, prolactin, and IGFBP1 levels were lower in RIF patients.
  \item Low PIBF1 expression may account for poor endometrial receptivity in RIF patients.
\end{itemize}

\textbf{Abstract}

Introduction: Recurrent implantation failure (RIF) is a challenging problem of assisted reproductive technology that arises mainly due to inadequate endometrial receptivity and its pathogenesis is still unclear. Objectives: In this study, we conducted the first investigation of the effect of decreased PIBF1 expression in mid-secretory phase on endometrial receptivity in patients with RIF. Methods: Microarray assay, reverse transcriptase-quantitative polymerase chain reaction, western blot, and in-vitro experiments were conducted. Results: The results showed that progesterone-induced blocking factor 1 (PIBF1) expression was highest in the mid-secretory endometrium in control subjects, but was significantly lower in RIF patients. In Ishikawa and human endometrial stromal cells (HESCs), rather than human endometrial epithelial cells, PIBF1 knockdown significantly downregulated cell proliferation and the levels of interleukin 6 (IL6) and
Introduction

Recurrent implantation failure (RIF), known as a failure to achieve clinical pregnancy after having undergone more than three embryo transfer cycles with the transfer of a total at least four good-quality embryos (score ≥ 7 or grade of 3BB or better) [1], is a difficult clinical issue in the area of in-vitro fertilization and embryo transfer (IVF-ET) [2]. Approximately 10% of sterile women receiving IVF-ET undergo RIF, which is associated with a substantial emotional and economic burden in these women [2]. To date, effective treatment for RIF has only rarely been achieved [3]. Endometrial receptivity and embryo quality were reported to be the main factors influencing the efficacy of embryo implantation [4], and inadequate endometrial receptivity was the main cause of RIF [5,6].

Decidualization is vital for the acquisition of endometrial receptivity during the window phase. Proper proliferation of endometrial stromal cells is necessary for decidualization, and attenuating proliferation inhibited decidualization and, hence, led to pregnancy failure [7]. For example, decreased expression of homeobox A10 (HOXA10), a known marker of endometrial receptivity, was related to the inhibition of stromal cell proliferation during decidualization and HOXA10-mutant mice had reduced fertility [8,9]. Notch1 has been reported to regulate cell proliferation and promote successful decidualization, and depletion of the Notch1 gene in mice led to fewer offspring [10,11].

Progesterone-induced blocking factor 1 (PIBF1), first found in progesterone-treated lymphocytes from fertile women [12], has a great effect on cell proliferation. For example, increased PIBF1 expression promoted tumor cell proliferation [13], and PIBF1 depletion caused mitotic arrest [14]. Some researchers have found that the lower PIBF1 expression was associated with abortion, preterm labor and pregnancy complications, while higher PIBF1 expression was the main cause of RIF [5,6].

Microarray assay

The mRNA expression profiles of three control and three individuals with RIF were analyzed using the Affymetrix GeneChip® PrimeView™ Human Gene Expression Array (Thermo Fisher Scientific, Meridian, USA). Data from the Gene Expression Omnibus (GEO) database (series GSE103465) were analyzed. We also analyzed data from a set of patients with RIF and 18 control subjects on day LH + 7 were collected and analyzed to compare the expression of PIBF1 and PIBF1 signaling relevant molecules (Table 3). Endometrium samples from 48 control subjects in all 6 phases of the menstrual cycle were collected for PIBF1-expression analysis (Table 4). What else, for isolation of primary endometrial cells, endometrium from RIF patients (mid-secretory phase, n = 11) and women with history of pregnancy who were acted as controls (late-proliferative phase, n = 15; mid-secretory phase, n = 15) were collected.
Complementary DNA (cDNA) preparation and quantitative real-time polymerase chain reaction (RT-qPCR) analysis

RNA extraction from endometrial biopsy tissues or cultured cells was performed according to published instruction (9767; Takara, Beijing, China). cDNA was synthesized using the reverse-transcription kit (RR036A, Takara, Beijing, China). The cDNA was frozen at −20 °C until use.

qPCR was performed as we described previously [21], and mRNA expression was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The 2−ΔΔCt method was applied to calculate the relative mRNA expression levels. The primer pairs used in this study are shown in Table 1.

Western blot immunoassay

Protein was isolated from samples using RIPA buffer (89900, Thermo, Meridian, USA) with 10% protease inhibitor (5892970001, Roche, Mannheim, Germany). 60 μg of protein was separated by 10% SDS-PAGE and transferred onto 0.22-μm polyvinylidene difluoride membranes at 90 mV for 90 min. After blocking, the membranes were incubated with primary antibodies (Table 2) for 12 h at 4 °C. The membranes were rinsed with Tris-buffered saline and incubated with 5% normal goat serum (room temperature, 30 min). Thereafter, the slides were incubated with anti-saline and incubated with 5% normal goat serum (room temperature, 30 min). Then, the slides were flushed with Tris-buffered saline and incubated with 5% normal goat serum (room temperature, 30 min). Thereafter, the slides were incubated with Tris-buffered saline and incubated with 5% normal goat serum (room temperature, 30 min). Subsequently, they were incubated with secondary antibodies, stained with diaminobenzidine, and counterstained with hematoxylin before visualizing the slides using the Nikon Eclipse 80i microscope.

IHC score of each section were used for analysis.

Pathologists performed the scoring independently, and the IHC scoring was randomly chosen under 400 magnification. Two

Immunohistochemical staining

Tissues were fixed with 4% paraformaldehyde (BL539A, Biosharp, Shanghai, China) and embedded in paraffin. Next, each section (thickness, 5-μm) was deparaffinized, rehydrated, subjected to antigen retrieval, and submerged in 3% hydrogen peroxide (10 min). Then, the slides were flushed with Tris-buffered saline and incubated with 5% normal goat serum (room temperature, 30 min). Thereafter, the slides were incubated with anti-PiBF1 (1:100), anti-Ki67 (1:200), or normal rabbit IgG antibodies at 4 °C (12 h). Subsequently, they were incubated with secondary antibodies, stained with diaminobenzidine, and counterstained with hematoxylin before visualizing the slides using the Nikon Eclipse 80i microscope.

The intensity of PiBF1 and Ki-67 staining was graded by calculating the immunohistochemical (IHC) score [21]:

IHC score = Σ (% of immunostained cells × intensity of staining). The staining intensity of PiBF1 and Ki-67 was graded from 0 to 3 (corresponding to negative, weak, moderate, and strong staining, respectively). A 600 × 600 pixel area within each slide used for scoring was randomly chosen under 400× magnification. Two pathologists performed the scoring independently, and the IHC scores of each section were used for analysis.

Culturing human endometrial stromal cells (HESCs), human endometrial epithelial cells (HEECs), and Ishikawa cells

Primary cells were separated from endometrial samples. First, the tissues were minced and digested for 1 h with 0.1% collagenase (171000017, Gibco, NY, USA) in a 37 °C shaker. Each mixture was then passed through a 100-μm sieve and then a 40-μm sieve. Converge and centrifuged the flushing and the reverse-flushing percolate from the 40-μm sieve to collect primary HESCs and HEECs, respectively. HESCs represent spindle-shaped fibroblast-like morphology while HEECs were polygonal cells and the purity

Table 1

| Primer | Sequence (5′-3′) |
|--------|-----------------|
| PiBF1-F | TTAGTGTCCGTGCTGTACAT |
| PiBF1-R | AATTGCTTCTGATGCTACAT |
| IL6-F | AGACAGATTAGTTACTGAGCAAC |
| IL6-R | GCCATTGTGTTGGTGTCCAG |
| MK67-F | GTGGAAGTTCTGCAACTGCA |
| MK67-R | TACGTCCAAAATTTTCAGCC |
| PRL-F | AGCGCAAGCCACACAGAGT |
| PRL-R | TACTCCGTGACAGAGGATACAG |
| IGFBP1-F | CCCAGAGACGCCACAGATAAC |
| IGFBP1-R | GGTGAATGAGAGACGGCTCC |
| GAPDH-F | TGCCTTCAACCGCCACACCC |
| GAPDH-R | CACGCTTGCTGTAGCCAAA |

Table 2

| Antibody | Dilution ratio | Application | Product details |
|----------|---------------|-------------|-----------------|
| Anti-PiBF1 rabbit polyclonal antibody | 1:1000 | Western blotting | ab183107, Abcam, CA, USA |
| Anti-IL6 mouse monoclonal antibody | 1:1000 | Western blotting | ab9324, Abcam, CA, USA |
| Anti-Phospho-STAT3 (Tyr705) rabbit monoclonal antibody | 1:1000 | Western blotting | #9145, Cell Signaling, MA, USA |
| Anti-STAT3 mouse monoclonal antibody | 1:1000 | Western blotting | #9139, Cell Signaling, MA, USA |
| Anti-HOXA10 goat polyclonal antibody | 1:1000 | Western blotting | ab191470, Abcam, CA, USA |
| Anti-PRL rabbit monoclonal antibody | 1:1000 | Western blotting | ab188229, Abcam, CA, USA |
| Anti-IGFBP1 rabbit monoclonal antibody | 1:1000 | Western blotting | ab180948, Abcam, CA, USA |
| Anti-GAPDH rabbit monoclonal antibody | 1:1000 | Western blotting | #5174, Cell Signaling, MA, USA |
| HRP-linked anti-rabbit IgG antibody | 1:5000 | Western blotting | #7074, Cell Signaling, MA, USA |
| HRP-linked anti-mouse IgG antibody | 1:5000 | Western blotting | #7076, Cell Signaling, MA, USA |
| HRP-linked anti-goat IgG antibody | 1:1000 | Western blotting | A0266, Beyotime, Shanghai, China |
| Anti-PiBF1 rabbit polyclonal antibody | 1:100 | Immunohistochemical staining | ab183107, Abcam, CA, USA |
| Anti-Ki67 rabbit polyclonal antibody | 1:200 | Immunohistochemical staining | ab16667, Abcam, CA, USA |
| Anti-Vimentin rabbit monoclonal antibody | 1:500 | Immunocytochemical staining | ab92547, Abcam, CA, USA |
| Anti-Cytokeratin 7 rabbit monoclonal antibody | 1:100 | Immunocytochemical staining | ab813598, Abcam, CA, USA |
| Goat polyclonal Secondary Antibody to Rabbit IgG - H&L (Alexa Fluor 488) | 1:500 | Immunocytochemical staining | ab150077, Abcam, CA, USA |
| Alexa Fluor® 594 GAR | 1:100 | Immunocytochemical staining | R37117, Invitrogen, OR, USA |
was > 95%. Cultured HESCs and HEECs were identified by immuno-

cytometry after staining with stromal cell marker vimentin (Table 2) or epithelial marker cytokeratin (Table 2) as described previously (Fig. S1) [21,22].

The cells were incubated in medium for two weeks containing DMEM/F12, 1% penicillin/streptomycin (15140122, Gibco, NY, USA), and 10% fetal bovine serum (10091141, Gibco, NY, USA) at 37 °C (5% CO₂).

**Lentiviral silencing and plasmid overexpression**

Short-hairpin RNA (shRNA) against human PIBF1 and negative shRNA were produced by Hanbio company (Shanghai, China), and PIBF1 and interluekin 6 (IL6) overexpression and mock plasmids were produced by GeneCopoeia (Guangzhou, China). The negative shRNA (5′-TTCTCGAACTGTCACGTAAA-3′) or PIBF1-shRNA (5′-TCTTGAGGAATTGGCAGCAATGAAA-3′) were transduced into cells (concentration, 2 × 10⁶ transducing units/mL) using polybrene (10 µg/mL). The cells were selected with puromycin (1 µg/mL) and then cultured with the puromycin reagent (0.5 µg/mL). The plasmids bought from GeneCopoeia were transfected into cells with transfection reagent (06366236001, Roche, Mannheim, Germany), following the manufacturer’s instructions.

**Proliferation assay**

Cell proliferation was assayed in Ishikawa cells and HESCs with the Cell Counting Kit (CCK-8; CK-04, Dojindo Molecular Technologies, Inc., Japan). The cells were plated in 96-well plates (1,000 cells/well), and CCK-8 solution (10 µl/well) was added on the second day after plating. The optical density (OD) was measured using a Multiskan GO instrument at 450 nm. The IL6 levels for each sample were normalized to the amount of the total protein in the cultured cells.

**In-vitro decidualization assay**

HESCs isolated from late proliferative-phase endometrium samples were isolated for analysis. After 4 days of lentivirus transduction and 3 days of plasmid transfection, the cells were then cultured in serum-free medium with β-estradiol (10 nM, E2758, Sigma, MO, USA), progesterone (1 µM, V900699, Sigma), and 8-Br-cAMP (1 mM, ab141448, Abcam, CA, USA) for 48 h. Total RNA was extracted and two markers of decidualization (pPRL and insulin-like growth factor-binding protein 1 [IGFBP1]) were analyzed with RT-qPCR and western blot. The primers and probes used are shown in Table 1 and Table 2, respectively. Each experiment was repeated at least three times.

**Enzyme-linked immunosorbet assay (ELISA) analysis**

IL6 secretion was measured using ELISA kits (ml058097, Milbio, Shanghai, China) with 50 µl culture medium, following the manufacturer’s instructions. The standard curves were plotted using recombinant human IL6. The OD was measured using a Multiskan GO instrument at 450 nm. The IL6 levels for each sample were normalized to the amount of the total protein in the cultured cells.

**Statistical analysis**

For continuous variables, the two-tailed Student’s t-test was applied to calculate differences between two groups. Differences across more than two groups were analyzed by analysis of variance (ANOVA) and the Bonferroni test for data with established normality and homogeneity of variance. Otherwise, a nonparametric test was applied to analyze the data. For analyzing categorical variables, the Fisher’s exact test was used. All data processing was managed using SPSS software (version 23.0, IBM, Inc.), and statistical significance was set at P < 0.05. All data related to the figures are displayed in Supplementary Table 2. The western blot images of in-vitro cell experiments in triplicates and the western blot images for endometrial tissues of all patients are provided in Supplementary Fig. S7-S14.

**Results**

**Patient characteristics**

Except for the number of embryo transfers, there was no significant difference in the characteristics between the control and RIF patients, or among patients during different phases of the menstrual cycle (Table 3-4).

**Microarray results**

According to the results of our previous microarray assay [1], 2519 DEGs displaying > 2-fold changes (adjusted P-value < 0.05) were identified in the RIF group. Among these genes, PIBF1 expression was lower in the RIF group (fold change = -2.15, P < 0.05, Fig. 1a). In addition, the gene-expression profiles of GSE58144 and GSE111974 showed that PIBF1 expression was lower in the RIF group (fold change = -1.06 and -1.21 respectively, P < 0.01, Fig. S2). In our microarray assay, Pearson correlation tests were carried out between PIBF1 and all genes detected. The top 300 genes with the highest correlations were screened and passed to us.

**Table 3**

Demographic characteristics of control subject and patients with RIF in mid-secretory phase recruited in this study.

| Variable               | Control (n = 18) | RIF (n = 20) | P     |
|------------------------|-----------------|--------------|-------|
| Age (y)                | 30.56 ± 3.45    | 30.50 ± 3.25 | 0.960 |
| BMI (kg/m²)            | 22.88 ± 3.52    | 21.63 ± 2.34 | 0.215 |
| Basal FSH level (mIU/mL)| 6.08 ± 1.35    | 6.85 ± 1.87  | 0.164 |
| Basal LH level (mIU/mL)| 4.19 ± 2.29    | 3.56 ± 2.16  | 0.946 |
| Basal E2 level (pg/mL) | 37.99 ± 13.56   | 40.50 ± 18.99| 0.646 |
| P₄ level on the day of ET (ng/mL) | 13.27 ± 6.72 | 16.61 ± 5.20 | 0.055 |
| P₄ level on the day of endometrial biopsy (ng/mL) | 15.40 ± 4.17 | 17.21 ± 3.14 | 0.137 |
| Endometrial thickness on the day of embryo transfer (mm) | 8.67 ± 0.39 | 8.47 ± 0.38 | 0.125 |
| Median embryo-transfer number (range) | 1 (1, 1) | 4 (3, 10) | < 0.001a |
| Number of embryos per transfer (%) | 9.094b |
| 1                      | 6/18 (33.3%)    | 31/97 (32.0%)|       |
| 2                      | 12/18 (66.7%)   | 66/97 (68.0%)|       |
| Median score of transferred day (range) | 8 (7, 9) | 8 (7, 10) | 0.564a |
| 3 embryos (range)      |                |              |       |
| Score of transferred blastocysts (%) | – |
| 3BB (/, /)             | 11/38 (19.0%)   |              |       |
| 4AB (/, /)             | 25/88 (41.3%)   |              |       |
| 4BB (/, /)             | 22/58 (37.0%)   |              |       |

The data are presented as the mean ± SD and were analyzed by Student’s t-test, unless otherwise stated. “*” levels on the day of ET > 40 ng/mL in the control (5 for the fresh embryo-transfer cycle and 2 for the frozen embryo-transfer cycle) and RIF (5 for the fresh embryo-transfer cycle and 8 for the frozen embryo-transfer cycle) groups were excluded. BMI, body–mass index; LH, luteinizing hormone; FSH, follicle-stimulating hormone; E₂, estradiol; P₄, progesterone. a, Mann–Whitney test; b, Fisher’s exact test
through the STRING database to construct a protein – protein interaction network. STAT3 was identified as a hub gene with a rather high node degree (Fig. 1b).

Characterization of PIBF1 expression in endometrial tissues and in HESCs after progesterone treatment

In the endometria of control subjects, the mRNA and protein levels of PIBF1 were low during the proliferative phases, increased in the early secretory phase, and peaked in the mid-secretory phase during the menstrual cycle (Fig. 2a–2c). Immunohistochemical staining revealed that PIBF1 was localized in the epithelium and stroma of the endometrial tissues (Fig. 2d). In the stroma, the staining intensity was lower in the RIF group compared to the control group (Fig. 2e, P < 0.01). In the epithelium, although the staining intensity showed a tendency towards decreased levels in the RIF group, no statistical difference was found (Fig. 2e). The mRNA and protein levels of PIBF1 in mid-secretory endometrium (Fig. 2f–2h, P < 0.01) and HESCs (Fig. 2i–2k) were notably lower in the RIF group compared to those in the control group. HOXA10 protein expression was significantly lower in the RIF group (Fig. 2g–2h, P < 0.01). In addition, PIBF1 expression in HESCs was induced by progesterone in a dose-dependent manner (Fig. S3a–b), and the levels of PIBF1, IL6, PRL, IGFBP1, p-STAT3 and STAT3 could be induced by progesterone or by the cocktail of estrogen, progesterone and cAMP (Fig. S3c–d).

Changes following knockdown of PIBF1 in Ishikawa cells, HESCs, and HEECs

Fig. 3a–3h revealed that the mRNA and protein levels of IL6, the protein levels of phosphorylated STAT3, and cell
proliferation decreased significantly in Ishikawa cells and HESCs following PIBF1 knockdown. However, in HEECs, the mRNA and protein levels of IL6 (Fig. 3i–3k) and the mRNA-expression level of MKI67 (Fig. 3l), a marker of proliferation, were not significantly changed after PIBF1 knockdown. Our ELISA results confirmed that, after PIBF1 knockdown, IL6 protein expression was considerably more decreased in HESCs ($P < 0.01$) than in HEECs ($P > 0.05$) (Fig. S4). In addition, measuring the in-vitro decidualization activity revealed that the mRNA and protein levels of PRL and IGFBP1 (markers of decidualization) and the protein levels of IL6 and p-STAT3 decreased following PIBF1 knockdown in HESCs (Fig. 3m–3o).

Changes following PIBF1 overexpression in PIBF1 shRNA-transduced Ishikawa cells and in HESCs and HEECs

Fig. 4a–4h shows that in Ishikawa cells transduced with negative-control or PIBF1 shRNA, as well as in HESCs isolated from control subjects and patients with RIF, IL6 and p-STAT3 production and cell proliferation increased notably following PIBF1 overexpression. The elevated levels of p-STAT3 and increased cell proliferation with PIBF-1 overexpression could be suppressed by inhibition of IL-6 (Fig. S5). However, in HEECs, the mRNA and protein levels of IL6 (Fig. 4i–4k) and mRNA levels of MKI67 (Fig. 4l) did not significantly change after PIBF1 overexpression.
Furthermore, the in vitro-decidualization activity assay revealed that the mRNA and protein levels of PRL and IGFBP1, as well as the protein levels of IL6 and p-STAT3, clearly increased in HESCs following PIBF1 overexpression (Fig. 4m–4o).

Changes following IL6 overexpression in PIBF1 shRNA-transduced Ishikawa cells and in HESCs and HEECs

Using Ishikawa cells transduced with negative-control and PIBF1 shRNA, as well as HESCs isolated from control subjects and RIF patients, we found that p-STAT3 production and cell proliferation increased notably following IL6 overexpression (Fig. 4m–4o).

In vivo expression of IL6, p-STAT3, PRL, IGFBP1, and Ki-67 in endometrial tissues

In the three microarray datasets above mentioned, IL6, PRL, and IGFBP1 were downregulated in the RIF group, except for the IL6 in GSE111974 and the PRL in GSE103465, which were slightly upregulated without significance (Fig. S2). Subsequent validation revealed that the mRNA and protein levels of IL6, PRL, and IGFBP1 were remarkably lower in the RIF group (Fig. 5a–6i). What else, the p-STAT3 level was lower in the RIF group (Fig. 6b–6c, \( P < 0.01 \)) and the staining intensity of Ki-67 was lower in the stroma of the RIF group (Fig. 6j–6k, \( P < 0.01 \)).
Implantation failure is a problematic clinical issue in reproductive medicine that is mainly related to inadequate endometrial receptivity, and no clear explanatory mechanisms have been purported [5,6]. In this study, we found that decreased PIBF1/IL6/p-STAT3 expression during the mid-secretory phase inhibited human endometrial stromal cell proliferation and decidualization, which may be one of the causes of implantation failure.

During the menstrual cycle, PIBF1 expression in the endometrium of the controls was relatively low in the proliferative phase, elevated in the early secretory phase, and peaked in the mid-secretory phase, suggesting that high PIBF1 expression during the implantation window helps the acquisition of endometrial receptivity, which may be one of the causes of implantation failure.

The mRNA-expression levels of PIBF1 and IL6 after PIBF1 overexpression in Ishikawa cells transduced with negative-control or PIBF1 shRNA were determined in HESCs and HEECs. Protein levels of PIBF1, IL6, phosphorylated STAT3 (Y705), and STAT3 in Ishikawa cells transduced with negative-control or PIBF1 shRNA and in HESCs isolated from control or RIF patients were also determined. Protein-expression levels of PIBF1 and IL6 after PIBF1 overexpression in HESCs were determined, as were the protein levels of PIBF1 and IL6 after PIBF1 overexpression in HEECs.

Previous studies have shown that PIBF1 could bind to the promoter of IL6 to enhance its expression [26] and that IL6 stimulated STAT3 phosphorylation to promote cell proliferation in many types of cancer cells [27–29]. Furthermore, prior research demonstrated that IL6 could induce the proliferation of stromal cells from patients with endometriosis by activating STAT3 [30]. In this study, our microarray analysis revealed that STAT3 belonged to the hub of PIBF1-related genes, and the IL6 and p-STAT3 levels decreased markedly in the RIF group, which corresponded with the PIBF1-expression changes in individual patients. Following PIBF1 knockdown, the proliferation and levels of IL6 and phosphorylated STAT3 decreased significantly in the RIF group during the mid-secretory phase, which was consistent with the expression of HOXA10, an accepted marker of endometrial receptivity [25]. These results implied that decreased expression of PIBF1 during the window phase may be one of the prime causes underlying the inadequate endometrial receptivity.
in Ishikawa cells and HESCs decreased significantly, which could be reversed by overexpressing PIBF1 or IL6. What else, the increased cell proliferation and elevated levels of p-STAT3 following PIBF-1 overexpression in Ishikawa cells transduced with negative-control or PIBF1 shRNA (a) and in HESCs isolated from control subjects or patients with RIF (f and g). Cell proliferation after IL6 overexpression in Ishikawa cells transduced with negative-control or PIBF1 shRNA (d) and in HESCs (h), as determined by the CCK-8 assay, and in HEECs (i), as determined by measuring the mRNA-expression level of MKI67, a marker of cell proliferation. The mRNA-expression levels (j) of PRL and IGFBP1 (decidualization markers) and protein levels (k and l) of PRL, IGFBP1, and phosphorylated STAT3 after IL6 overexpression during in vitro decidualization. HESCs were isolated from control subjects and patients with RIF as indicated (f and g). NC, negative control; PIBF1-KD: PIBF1-knockdown group; IL6-OE: IL6-overexpression group. GAPDH and STAT3 were detected as loading controls. All data are presented as the mean ± SD. *P < 0.05, **P < 0.01.

Fig. 5. Changes in IL6 expression, STAT3 phosphorylation, cell proliferation, and stromal cell decidualization following IL6 overexpression. IL6 mRNA expression after IL6 overexpression in Ishikawa cells transduced with negative-control or PIBF1 shRNA (a) and in HESCs (e) and HEECs (i). Protein levels of IL6, phosphorylated STAT3 (Y705), and STAT3 in Ishikawa cells transduced with negative-control or PIBF1 shRNA (b and c) and in HESCs isolated from control subjects or patients with RIF (f and g). Cell proliferation after IL6 overexpression in Ishikawa cells transduced with negative-control or PIBF1 shRNA (d) and in HESCs (h), as determined by the CCK-8 assay, and in HEECs (i), as determined by measuring the mRNA-expression level of MKI67, a marker of cell proliferation. The mRNA-expression levels (j) of PRL and IGFBP1 (decidualization markers) and protein levels (k and l) of PRL, IGFBP1, and phosphorylated STAT3 after IL6 overexpression during in vitro decidualization. HESCs were isolated from control subjects and patients with RIF as indicated (f and g). NC, negative control; PIBF1-KD: PIBF1-knockdown group; IL6-OE: IL6-overexpression group. GAPDH and STAT3 were detected as loading controls. All data are presented as the mean ± SD. *P < 0.05, **P < 0.01.

During the menstrual cycle, PIBF1 expression in the endometrium is consistent with the levels of progesterone, and our in vitro experiment showed that PIBF1 expression increased in dose-dependent manner after progesterone treatment in HESCs. Further study proved that both progesterone and the cocktail of decidualization containing progesterone could induce the expression levels of PIBF1/IL6/p-STAT3 in HESCs, suggesting the effect of progesterone on the induction of this pathway. However, levels of PIBF1/IL6/p-STAT3 decreased significantly while the serum progesterone level on the day of biopsy was not reduced in the RIF group. These results implied that the decreased expression of PIBF1 in RIF patients during mid-secretory phase was affected by other factors besides progesterone. For example, some groups reported that PIBF1 expression was also affected by the IL-33 concentration, as well as the number of B cells and mitochondrial DNA copies [15,36,37]. The reason(s) underlying PIBF1 downregulation in patients with RIF should be further investigated.
Conclusion

In summary, we found that PIBF1 expression changed dynamically during the menstrual cycle and peaked in mid-secretory phase. During the same period, in the endometrium of RIF group, the levels of PIBF1, IL6, and p-STAT3 decreased significantly, and the proliferation and decidualization of HESCs were notably inhibited. Our results suggested that lower PIBF1 expression inhibited the proliferation and decidualization of HESCs by decreasing the levels of IL6 and p-STAT3, which could be one of the main causes of poor endometrial receptivity in RIF patients. However, further study on the mechanism of decreased PIBF1 levels in the mid-secretory endometrium and the mechanism by which PIBF1 may regulate endometrial receptivity should be conducted with animal models.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2020.09.002.

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