MiR-133b Improves Decidualization of Endometrial Stromal Cells by Targeting KLF12 in Recurrent Implantation Failure

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Abstract

Purpose Impaired decidualization contributes to the infertility in recurrent implantation failure (RIF). Herein, we focused on the function and probable mechanisms of miR-133b in endometrial stromal cells decidualization.

Methods miR-133b and KLF12 protein levels in midsecretory endometrial tissues derived from women with and without RIF were measured by qRT-PCR and Western blot. Primary human endometrial stromal cells (HESCs) were isolated and cultured for in vitro decidualization assays. Luciferase reporter, qRT-PCR and Western blot assays were used to measure the relationship between miR-133b and KLF12.

Results miR-133b was significantly downregulated, whereas KLF12 was upregulated in endometrial tissues from RIF. miR-133b effectively promoted HESCs in vitro decidualization through the modulation of KLF12 expression and the activation of LIF/STAT3 pathway. Conversely, inhibition of miR-133b expression reversed these effects. In addition, the luciferase reporter system demonstrated that miR-133b directly inhibited the expression of KLF12 by interacting with 3' untranslated region of KLF12.

Conclusion Our data suggest that miR-133b promotes HESCs decidualization by targeting KLF12 and reverses the impaired decidualization in RIF.

Introduction

Recurrent implantation failure (RIF), one of the common causes of infertility for female undergoing in vitro fertilization (IVF) treatment, is disastrous for both couples and clinicians[1]. Although there is no consensus in the definition of RIF, it can be defined as failure to achieve a clinical pregnancy after three or more transfers of high-quality embryos or the transfer of 4 high-quality embryos in multiple cycles[2]. In order to be suitable for embryo implantation, a receptive endometrium allows the adhesion and invasion of an embryo during the window of implantation (WOI)[3]. However, impaired endometrial receptivity and disrupted decidualization of endometrium lead to embryo implantation failure[4, 5].

Decidualization, characterized by the transformation of human endometrial stromal cells (HESCs) into specialized secretory decidual cells, is the necessary process for blastocyst invasion[6–8]. HESCs undergo vast expansion and differentiation after progesterone (P4)-mediated the activation of cyclic AMP signaling[7, 9]. Successful implantation and pregnancy maintain depend on stromal cells decidualization which has been demonstrated to play important roles in reproductive disorders such as RIF[10] and recurrent miscarriage[11]. Mechanistically, leukemia inhibitory factor (LIF), a member of IL-6 family, plays a vital role in the progression of decidualization and embryo to implantation[12]. What's more important, LIF-mediated STAT3 activation by promoting the phosphorylation of STAT3 to regulate the dialogue between endometrium and embryo[13–15]. Especially, the LIF/STAT3 pathway is dysregulated in RIF[16]. However, the precise mechanisms underlying the process of decidualization in RIF need further exploration.

microRNAs, well known as evolutionarily conserved single-stranded and small noncoding RNA molecules with 20–22 nucleotides, regulate the target genes expression by binding to the 3'-untranslated region (3'UTR) of mRNAs and play important roles in the posttranscriptional regulation of physiological process[17]. Numerous of evidence has shown the important of miRNAs in the development of obstetrics and gynecology diseases, including preeclampsia[18], endometriosis[19] and recurrent implantation failure[20]. Furthermore, multiple studies suggest that miRNAs play important roles in endometrial receptivity and decidualization[7, 19]. In our previous study, miR-133b reverses the hydrosalpinx-induced impairment of endometrial receptivity[21]. However, the role of miR-133b in decidualization is still largely unknown.

In this study, we found that miR-133b contributes to endometrial decidualization by activating the LIF/STAT3 pathway and directly targeting KLF12 expression. Moreover, the level of miR-133b was significantly decreased in the endometrium of RIF, while KLF12 was upregulated. This study provides initial evidence that miR-133b is critical for decidualization and may be a promising therapeutic target for RIF.

Materials And Methods

Participants and tissue collection

The use of human tissues was approved by the Ethical Committee of Changzhou Maternal and Child Health Care Hospital Affiliated to Nanjing Medical University. All participants signed a written consent form before participating in the study.

The participants who underwent in vitro fertilization (IVF) involved in this study were recruited from August 2018 to January 2020. A total of 12 patients without achieving clinical pregnancy after at least four good-quality embryo transfers in a minimum of three fresh or frozen cycles under the age of 40 years were enrolled as the RIF group. The control group consisted of 12 women who achieved live birth within the first three transfer cycles. Women with autoimmune disturbances, endocrine dysfunctions, diminished ovarian reserve, polycystic ovarian syndrome (PCOS), endometrial hyperplasia, endometrial polyps, hydrosalpinx, endometriosis, adenomyosis or intrauterine adhesions were excluded. The details of these participants are summarized in Table 1. Endometrial samples were derived from the endometrial biopsy between days 19 and 23 of menstruation from all women.

Table 1

Demographic details of the participants in this study

Fertile	FER (n=12)	RIF (n=12)	P
Age (years)	29.9 ± 3.2	30.5 ± 3.2	ns
Body mass index (Kg/m ²)	20.7 ± 1.8	21.1 ± 1.5	ns
Menstrual cycle (days)	29.9 ± 2.5	29.5 ± 3.9	ns
Endometrial thickness (mm)	10.2 ± 1.0	9.7 ± 1.1	ns
No. of transferred embryos	2 ± 0	8.6 ± 4.0	s
The data are presented as the mean ± SD unless otherwise indicated			

Isolation and culture of primary HESCs

Primary HESCs were isolated from endometrial tissues according to a previously described method[19]. After purification, HESCs were digested and grown into new plates with DMEM/F12 (HyClone, Thermo Scientific, South Logan, UT, USA), 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 1% penicillin and 1% streptomycin. The cells were used to induce decidualization as described previously.

Construction of adenoviruses

Adenoviruses harboring a DNA fragment encompassing the miR-133b gene or full-length KLF12 were generated using the AdMax (Microbix Biosystems, Inc., Toronto, Canada) system. The viruses were packaged, amplified and purified according to a previously protocol.

Western blotting

The proteins extracted from endometrial tissues and cultured cells as previously described[21]. After using the BCA kit (Beyotime, Shanghai, China) to measure the protein concentration, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate the proteins, which were then blotted onto polyvinylidene difluoride (PVDF, Millipore, Billerica, MA, USA) membranes and incubated with the primary antibodies including anti-KLF12 (Santa Cruz Biotechnology, 1:1000), anti-LIF (Abcam, 1:500), anti-STAT3 (Cell Signaling Technology, 1:1000), anti-p(Y705)-STAT3 (Santa Cruz Biotechnology, 1:1000) or anti-β-actin (Abcam, 1:5000), followed by incubations with a horseradish peroxidase (HRP)-conjugated secondary antibody or Flag-HRP. The bands were visualized with an enhanced chemiluminescence kit (Amersham Biosciences Corp., Piscataway, NJ, USA) in ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA).

Quantitative Real-time PCR

Total RNA was extracted from endometrial tissues or HESCs using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. cDNA was synthesized by using a PrimeScript RT reagent kit (Takara). Realtime-PCR were employed with SYBR Green kit (Qiagen, Hilden, Germany) on a MyiQ single color Realtime PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The level of RNA was calculated using the $2^{-\Delta\Delta C_t}$ method. 18S rRNA and U6 served as endogenous controls for mRNA and microRNA, respectively. The specific primers used for quantitative PCR analysis were: miR-133b, F: 5'-ACACTCCAGCTGGGTTTGGTCCCCTTCAAC-3' and R: 5'-GGTGTCTGTCGGAGTCGGCAATTCAGTTGAG-3'; U6, F: 5'-CTCGCTTCGGCAGCACA-3' and R: 5'-AACGCTTCACGAATTTGCGT-3'; KLF12, F: 5'-CCTTTCCATAGCCAGAGCAG-3' and R: 5'-TTGCATCCCTCAAATCACA-3'; LIF, F: 5'-AGTCGTGACCTTGGCACCTC-3' and R: 5'-GTTGACAGCCCAGCTTCTTC-3'; PRL, F: 5'-CACTACATCCATAACCTCTC-3' and R: 5'-ATGCTGACTATCAAGCTCAG-3'; IGFBP-1, F: 5'-TATGATGGCTCGAAGGCTCTC-3' and R: 5'-GTAGACGCACCAGCAGAGTC-3'; 18S rRNA, F: 5'-CGGCTACCACATCCAAGGAA-3' and R: 5'-CTGGAATTACCGCGGCT-3'.

Prolactin measurement in cultured supernatants

Prolactin levels in cell culture media were measured using the Mini-Vidas V.B02.96 System with a Vidas prolactin Kit (bioMerieux, France). Each experiment was performed in triplicates and repeated in three primary HESCs derived from different women.

Dual-luciferase reporter assay

The wild-type KLF12 3'-UTR (KLF12 3'-UTR WT) and mutant KLF12 3'-UTR (KLF12 3'-UTR MUT) were synthesized and inserted into the pGL3 luciferase reporter vector (Promega, Madison, WI, USA). For reporter assays, the preconfluent (70%) HEK293T cells in 24-well plates were transfected with the indicated plasmids using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA). The dual-luciferase reporter assay was performed to analyze the relative luciferase activity after transfection for 48 h.

Statistical analysis

All statistical analysis were performed using Prism version 8 software (GraphPad, LaJolla, CA). The data were presented as the mean $\pm$ SEM of at least three independent experiments. Student's t-test was used for comparisons between two groups. One-way ANOVA was performed for comparisons among more than two groups. Pearson correlation analysis was used to assess the relationship between miR-133b and KLF12. Differences at $p < 0.05$ were considered significant.

Results

miR-133b promotes HESCs decidualization in vitro

As shown in Fig. 1a, the level of miR-133b was markedly increased in HESCs treated with 8Br-cAMP and MPA in a time-dependent manner. After confirming the enhanced expression of miR-133b adenovirus in HESCs (Fig. 1b), we investigated the role of miR-133b in HESCs decidualization. Overexpression of miR-133b promoted the mRNA levels of PRL, IGFBP-1 and LIF in HESCs treated with 8Br-cAMP and MPA (Fig. 1c, d, e). Moreover, ectopic expression of miR-133b contributed to increased decidual dPRL secretion during HESCs decidualization in vitro (Fig. 1f). Additionally, miR-133b overexpression significantly increased the protein levels of LIF and its downstream protein p-STAT3, which is the active form of STAT3 (Fig. 1g).

Inhibition of miR-133b attenuates the decidualization of HESCs

To investigate whether miR-133b inhibition attenuates 8Br-cAMP and MPA-induced HESCs decidualization in vitro, a synthesized anti-sense oligonucleotide of miR-133b (miR-133b inhibitor) was employed in HESCs (Fig. 2a). Transfection of the miR-133b inhibitor decreased the PRL, IGFBP-1 and LIF mRNA levels in HESCs treated with 8Br-cAMP and MPA (Fig. 2b, c, d). Moreover, the inhibition of miR-133b in HESCs led to decreased decidual dPRL secretion (Fig. 2e). In addition, the LIF/STAT3 signaling activation was decreased after miR-133b expression was inhibited in HESCs (Fig. 2f).

miR-133b directly targets KLF12 in HESCs

We cotransfected cells with wide-type KLF12 or mutant KLF12 along with pSilencer-CMV-miR-133b or empty vector and then employed dual-luciferase reporter assay to demonstrate whether KLF12 is a direct target of miR-133b. As shown in Fig 3a, KLF12 mRNA contained two putative target sites in its 3' Untranslated Region (3'UTR) that aligned with the seed sequence of miR-133b. Luciferase reporter assays indicated that overexpression of miR-133b markedly inhibited the activity of the wild-type KLF12 3'UTR luciferase reporter (Fig. 3b). Furthermore, overexpression of miR-133b markedly decreased the protein level of KLF12 (Fig. 3c), while inhibition of miR-133b in HESCs significantly increased KLF12 expression (Fig. 3d). These data suggested that KLF12 is a direct target of miR-133b.

KLF12 reverses the miR-133b-induced decidualization in HESCs

Next, we hypothesized that miR-133b enhanced HESCs decidualization through inhibiting KLF12 expression and impairing KLF12-mediated signaling activation. After confirming the enhanced expression of KLF12 adenovirus in HESCs (Fig.4a), we found that overexpression of KLF12 led to decreased LIF

mRNA and protein expression and suppressed LIF/STAT3 signaling activation (Fig. 4b, c). In addition, the mRNA levels of PRL and IGFBP-1 were markedly decreased in HESCs after KLF12 expression was enhanced (Fig. 4d, e). Importantly, forced overexpression of KLF12 in HESCs inhibited miR-133b-enhanced 8Br-cAMP and MPA-induced PRL, IGFBP-1 and LIF mRNA expression (Fig. 4f, g, h). In addition, adenovirus mediated KLF12 overexpression in HESCs repressed miR-133b-induced the LIF/STAT3 signaling activation (Fig. 4i). These data suggested that miR-133b promotes HESCs decidualization by regulating KLF12 expression and reversing KLF12-mediated LIF/STAT3 signaling inactivation.

Aberrant expression of miR-133b and KLF12 in the endometrial of patients with RIF

Because KLF12 disrupts the HESCs decidualization in patients with RIF, we further examined the mRNA level of miR-133b and protein expression of KLF12 in midsecretory endometrial tissues from patients with RIF. Analysis of miR-133b expression revealed that women with RIF had a lower (approximately 50%) miR-133b mRNA level relative to fertile controls (Fig. 5a). Conversely, expression of KLF12 was significantly higher (approximately 2-fold) in patients with RIF than in fertile controls (Fig. 5b). As shown in Fig. 5c, the miR-133b expression was negatively correlated with the protein expression of KLF12 ($r=-0.6962$, $p=0.0002$), consistent with the notion that miR-133b regulated KLF12 expression.

Discussion

RIF remains an extremely frustrating condition for both clinicians and patients[1]. Impaired endometrial receptivity and decidualization are considered as key factors in affecting embryo implantation in RIF patients. In this study, we demonstrated that decreased miR-133b expression inhibited endometrial decidualization in RIF patients. Besides, miR-133b promoted endometrial decidualization by targeting KLF12 expression and regulating STAT3/LIF signaling pathway.

Emerging evidence suggests that microRNAs play important roles in endometrial decidualization and embryo implantation[22]. Our previous study has found that miR-133b reverses the hydrosalpinx-induced defective endometrial receptivity by promoting HOXA10 expression in endometrial epithelial cells[21]. In addition, except participating in cell apoptosis, migration and invasion[23, 24], miR-133b has previously been implicated in myogenic differentiation program[25]. Interestingly, we found that miR-133b promoted HESCs decidualization, one type of cell differentiation, which is essential for reproduction[21]. Otherwise, our data showed that miR-133b was down-expression in RIF patients versus controls. These findings indicated that miR-133b might be a regulator for decidual response in women with RIF.

microRNAs are known to exert their biological functions via binding to the 3'-UTR of target genes. Here, KLF12 was experimentally examined as a direct target of miR-133b. Krüppel-like factor 12 (KLF12), a zinc finger-containing transcription factor, is expressed in glandular epithelium and stromal cells and takes part in steroid hormone signaling[19, 26]. Hui Zhang et al. has demonstrated that KLF12 impairs

endometrial decidualization via the transcriptional repression of FOXO1 both in vitro and in vivo[27]. In addition, similar to the research from Hui Zhang et al[27], we also found that excessive expression of KLF12 in midsecretory endometrial tissues from patients with RIF. Simultaneously, our results showed that the expression of miR-133b and KLF12 were highly negatively correlated in midsecretory endometrial tissues. After demonstrating that miR-133b targeted the 3'UTR of KLF12 and inhibited KLF12 expression, we found that adenovirus-induced overexpression of KLF12 abolished miR-133b-promoted 8Br-cAMP plus MPA-induced decidualization. These data demonstrated that miR-133b enhanced HESCs decidualization through targeting the negative decidualization regulator KLF12.

Leukemia inhibitory factor (LIF), a pro-inflammatory cytokine, plays a vital role in the progression of blastocysts to implantation[13]. *Lif* knockout mice show complete implantation failure with poor endometrial receptivity and decidualization[28]. Studies have demonstrated that aberrant expression of LIF in the endometrial tissue from infertile women impair the clinical pregnancy rate of the IVF-ET cycle[29]. Mechanistically, in endometrial luminal epithelial cells, LIF combines with the LIF receptor complex to form a stable LIF-LIFR-gp130 complex, which can activate JAK1 enzymes and increase the phosphorylation of the latent signal transducer and activator of transcription 3 (STAT3)[30]. Moreover, Youngsok Choi, et al. has demonstrated that LIF/STAT3 signaling is aberrantly reduced in uterine stromal cells of patients with RIF[16]. Chengyang Huang, et al. has further demonstrated that KLF12 binds to the sequence of "CAGTGGG" in the promoter region of LIF and represses the LIF/STAT3 signaling activation[31]. Similarly, we showed that KLF12 inhibited LIF/STAT3 signaling during HESCs decidualization. Besides, miR-133b promoted decidualization through targeting KLF12 expression and regulating LIF/STAT3 activation in vitro. However, whether miR-133b-KLF12-LIF-STAT3 axis plays a role in endometrial decidualization in vivo deserves further investigation.

In conclusion, our data show that miR-133b may promote HESCs decidualization through targeting KLF12 and indicate the aberrantly decreased miR-133b expression in the endometrium of women with RIF. Our study suggests that miR-133b may be a potential target in RIF with defective decidualization.

Declarations

Funding

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Author contribution

FL Mei, CC Kong and Y Wang contributed equally to this work. FL Mei designed the study. FL Mei, CC Kong, J Zhang, PP Xue, H Qi and Y Wang were responsible for acquisition of data. FL Mei and CC Kong drafted the manuscript. YS Dong performed the Formal analysis. All authors read and approved the final version of the manuscript.

Availability of data and material

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflicts of interest

The authors declare that they have no conflict of interest

Ethics approval

All procedures performed in this study involving human participants were in accordance with the ethical standards of the Ethical Committee of Changzhou Maternal and Child Health Care Hospital Affiliated to Nanjing Medical University and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

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Figures

Figure 1

miR-133b promotes HESCs decidualization in vitro. a The expression pattern of miR-133b in HESCs after induction of decidualization for different periods of time was determined by qRT-PCR. b HESCs were infected with Ad-miR-133b or Ad-LacZ at the indicated MOI. The expression of miR-133b was determined by qRT-PCR. HESCs were infected with Ad-miR-133b or Ad-LacZ at the indicated MOI for 24 h followed by treatment with 8Br-cAMP and MPA for 3 days. Prolactin (c), IGFBP-1 (d) and LIF (e) mRNA levels were measured by qRT-PCR; Prolactin released into the medium (f) was measured by enzyme-linked fluorescent assay. g The protein levels of LIF, STAT3 and p-STAT3 in HESCs infected with Ad-miR-133b or Ad-LacZ at the indicated MOI for 48 h were assessed by Western blot. *P < 0.05, **P < 0.01.

Figure 2

miR-133b downregulation attenuates the decidualization of HESCs. a HESCs were transfected with miR-133b inhibitors or control inhibitors at the indicated nM. The expression of miR-133b was determined by qRT-PCR. HESCs were transfected with miR-133b inhibitors or control inhibitors at the indicated nM for 24 h followed by treatment with 8Br-cAMP and MPA for 3 days. Prolactin (b), IGFBP-1 (c) and LIF (d) mRNA levels were measured by qRT-PCR; Prolactin released into the medium (e) was measured by enzyme-linked fluorescent assay. f The protein levels of LIF, STAT3 and p-STAT3 in HESCs transfected with miR-133b inhibitors or control inhibitors at the indicated nM for 48 h were assessed by Western blot.

Figure 3

miR-133b directly targets KLF12 in HESCs. a The putative binding sites for miR-133b in the 3'UTR of KLF12 mRNA. b Relative luciferase activity in H293T cells cotransfected with pSilencer-CMV-miR-133b or empty vector, and mutant or wild-type 3'UTR KLF12 was determined by dual-luciferase reporter assay. HESCs were treated with Ad-miR-133b (c) or miR-133b inhibitor (d) for 48 h. KLF12 protein levels were measured by Western blot.

Figure 4

KLF12 reverses the biological function of miR-133b in HESCs. HESCs were infected with Ad-Flag-KLF12 or Ad-LacZ at the indicated MOI for 48 h. The expression of KLF12 (a) and LIF (b) mRNA were determined by qRT-PCR; the protein levels of LIF, STAT3 and p-STAT3 were measured by Western blot (c). HESCs were infected with Ad-Flag-KLF12 or Ad-LacZ at the indicated MOI for 24 h followed by treatment with 8Br-cAMP and MPA for 3 days. Prolactin (d) and IGFBP-1 (e) mRNA levels were measured by qRT-PCR. HESCs were infected with Ad-LacZ, Ad-miR-133b, or miR-133b together with Ad-Flag-KLF12 for 24 h followed by treatment with 8Br-cAMP and MPA for 3 days. Prolactin (f), IGFBP-1 (g) and LIF (h) mRNA levels were measured by qRT-PCR. i HESCs were infected with Ad-LacZ, Ad-miR-133b, or miR-133b together with Ad-Flag-KLF12 for 48 h. LIF STAT3 and p-STAT3 protein levels were measured by Western blot.

Figure 5

Aberrant expression of miR-133b and KLF12 in the endometrial of patients with RIF. a Midsecretory endometrial tissues from fertile control (n=12) and patients with RIF (n=12) were analyzed for miR-133b expression by RT-PCR. b Midsecretory endometrial tissues from fertile control (n=12) and patients with RIF (n=12) were analyzed for KLF12 expression by Western blot. The total KLF12 protein levels of the FER and RIF tissues were normalized to β-actin. c Correlation between miR-133b mRNA levels and KLF12 protein expression ($r=-0.6962$, $p=0.0002$). $**p < 0.01$.