Expression and significance of miR-155 during embryo implantation

Jin-Chun Lu (✉ 406646227@qq.com)

Research article

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Abstract

Background

miRNAs are considered to be one of the important molecules involved in the regulation of multiple physiological functions of endometrium such as proliferation and decidualization, angiogenesis, immune regulation, adhesion and invasion. miR-155 is closely related to the occurrence of some diseases such as inflammation, tumor metastasis and immunity. In view of the extreme similarity in immune escape, angiogenesis, cell proliferation, differentiation and invasion between embryo implantation and tumorigenesis, we studied the role of miR-155 in embryo implantation.

Methods

Human endometrial tissues and decidual tissues were collected, and the mouse models of early pregnancy were constructed. The expression levels of miR-155 were analyzed by quantitative PCR.

Results

The expression levels of miR-155 in decidual tissues were significantly lower than that of endometrial tissues in the middle secretory stage ($P < 0.05$). The results of mouse models showed that the endometrial decidualization progressed gradually with the increase of gestational days, and that the expression levels of miR-155 in endometrial tissues decreased gradually with the increase of gestational days ($P < 0.05$). On day 5 of pregnancy, the expression levels of miR-155 in endometrial tissues of embryo implantation sites were significantly lower than that in inter-implantation sites ($P < 0.05$). The amount of embryo implantation in the uterus injected by mmu-miR-155 agomir was significantly lower than that by normal control agomir ($P < 0.05$).

Conclusions

During the embryo implantation of early pregnancy, the expression level of miR-155 in endometrial tissues decreased significantly with the increase of pregnancy time, which may be related to endometrial decidualization.

Introduction

Although assisted reproductive technology (ART) is making continuous progress and embryo quality is significantly improved, there are still data showing that more than 50% of pregnancy failure are caused by embryo implantation failure [1]. During embryo implantation, the morphology and structure of endometrium will change, and the secretory function of cells in endometrium will also increase significantly under the regulation of hormones. The cells in endometrium are able to secrete a lot of

adhesion molecules, cytokines, growth factors, lipids and other related molecules [2, 3], which play an important role in embryo implantation. An in-depth understanding of the molecular pathways involved in embryo implantation is of great significance to improve the diagnosis and treatment of infertility caused by poor implantation or decidualization.

In recent years, microRNA (miRNA) as a transcriptional regulator of gene expression was reported to be involved in the process of embryo implantation [4]. miRNA is a small non-coding RNA in eukaryotes, with a length of 22 nucleotides. miRNA can degrade mRNA or inhibit the translation level of mRNA by binding with specified mRNA [5, 6]. miRNAs are considered to be one of the important molecules involved in the regulation of multiple physiological functions of endometrium such as proliferation and decidualization, angiogenesis, immune regulation, adhesion and invasion [7]. miR-155 is located on chromosome 21q21.3 and is closely related to the occurrence of some diseases such as inflammation, tumor metastasis and immunity [8]. In view of the extreme similarity in immune escape, angiogenesis, cell proliferation, differentiation and invasion between embryo implantation and tumorigenesis, we studied the role of miR-155 in embryo implantation. We first compared the expression levels of miR-155 in endometrial tissues of the middle secretory stage of menstrual cycle and decidual tissues of early pregnancy, and further investigated the expression pattern of miR-155 before and after embryo implantation and the effect of abnormal expression of miR-155 on embryo implantation in the mouse model of early pregnancy.

Materials And Methods

Research subject and grouping

Six samples of endometrial tissues in the middle secretory stage of menstrual cycle were collected from the patients performed in vitro fertilization - embryo transplantation (IVF-ET) in the Center for Reproductive Medicine of our hospital due to male factors or fallopian tube factors during May 2017 and October 2017. At the same time, 6 samples of decidual tissues were collected from the women undergone the induced abortion in the family planning operating room of our hospital due to social factors. All tissues were quickly frozen at -80°C except for the pathological examination.

The inclusion criteria of endometrial tissues in the middle secretory stage of menstrual cycle were as follows. The collection time of endometrial tissues was determined by the results of ultrasound monitoring ovulation and sex hormones, that is, the endometrial tissues were obtained by curettage under sterile conditions on LH + 7-9 days. The pathological staining of endometrial tissues met the Noyes standard of metaphase's endometrium [9]. Normal decidual tissues came from the naturally conceived women with 7-8 weeks of gestation, and gestational sac and fetal heart beats could be observed under the ultrasound. Moreover, the pathological examination confirmed that the intimal tissues obtained by induced abortion were decidual tissues.

This study was approved by the ethics committee of our hospital, and the patient's informed consent was obtained.

Determination of miR-155 in metaphase's endometrium and decidual tissues

The collected intimal tissues were washed with sterile normal saline and divided into the tissue block with the size of about 1 cm × 1 cm × 1 cm. Partial tissues were fixed for the pathological examination, and the rest were stored in liquid nitrogen. The expression levels of miR-155 in intimal tissues, including metaphase's endometrium and decidual tissues, were detected by quantitative RT-PCR (qRT-PCR).

Construction of the mouse model of early pregnancy

The experimental ICR mice, aged 6-8 weeks and weighing about 25-30 g, were purchased from the Experimental Animal Center of Nanjing Medical University. The female mice in estrous stage were mated with the male mice in the ratio of 2:1. Seen vaginal plug was set as day 1 (D1) after coitus. The mice on Day 5 were injected with 0.1 ml of 1% Evans blue staining solution (Biosharp, China) into the tail vein to distinguish the embryo implantation site (IS) and inter-implantation site (IIS) in the mouse uterus. The implantation site was stained blue and the inter-implantation site was not. The mice were killed by cervical dislocation at 6:00 p.m. on the same day.

Collection and pathological staining of endometrial tissues from early pregnant mice

The uterus tissues of pregnant mice on D1, D4, D5 (including D5IS and D5IIS) and D6 were collected. Some tissues were fixed in 4% of paraformaldehyde and used for HE staining (HE staining kit, Baihao, China), and endometrial tissues were extruded from the rest tissues by the rolling method under sterile conditions. The endometrial tissues on D5IS and D5IIS was obtained by separating embryo and uterus tissues under a microscope. All obtained tissues were quickly frozen at -80°C for the detection of miR-155.

Detection of miR-155 in endometrial tissues by qRT-PCR

The total RNAs of human or mouse endometrial tissues were extracted by the Trizol method, and the concentration of total RNAs was measured by spectrophotometer. RNAs were transcribed into cDNA with specific primers for miR-155 and U6 (GenePharma, Shanghai, China) and further amplified by the real-time quantitative PCR. The relative expression levels of miR-155 were calculated by the $2^{-\Delta\Delta C_t}$ method.

Injection of mmu-miR-155 agomir and counting of embryos

Mice on D3 were anesthetized with 4% of newly prepared chloral hydrate at 14:00 p.m., and then bilateral ovaries and uterine horns were exposed. Twenty microliters of mmu-miR-155 agomir (1 nmol, GenePharma, Shanghai, China) were injected into the uterine cavity from the left uterine horn near the ovary (experimental side) with 50 μ l of microinjector. Normal control (NC) agomir of equal concentration and volume was injected into the right uterine horn near the ovary (control side) in the same way. These mice were killed by cervical dislocation at 6:00 p.m. on D8, and embryos on both sides were counted.

Statistical analysis

The SPSS 20.0 and GraphPad Prism 5 software were used for statistical analysis. The measurement data were expressed as mean $\pm$ standard deviation (SD). Comparisons between groups were assessed by one-way ANOVA or nonparametric tests, and the comparison of two groups by the paired *t* test. *P*-value < 0.05 was considered statistically significant.

Results

General clinical data

General information of 6 patients provided the metaphase's endometrial tissues and 6 women provided the decidual tissues were presented in Table 1 and Table 2.

Table 1
Basic parameters of 6 patients provided the metaphase's endometrial tissues

No. of patients	Age (years old)	No. of gestation	Menstrual cycle (days)	Estradiol in window period (pmol/L)	Progesterone in window period (nmol/L)
1	33	2	28	746	61.33
2	31	1	30	488	43.09
3	28	1	29	518	41.23
4	29	1	30	534	57.63
5	26	1	28	615	48.92
6	30	2	30	497	40.15

Table 2
Basic parameters of 6 women provided decidual tissues

No. of women	Age (years old)	No. of gestation	Menelipsis (days)	Are there fetal heart beats?	Human chorionic gonadotropin (IU/L)
1	26	1	54	Yes	75467
2	32	1	54	Yes	66342
3	29	1	52	Yes	52640
4	25	1	56	Yes	88763
5	27	1	48	Yes	40054
6	32	2	49	Yes	34387

Expression levels of miR-155 in metaphase's endometrial tissues and decidual tissues

The relative expression levels of miR-155 in decidual tissues were significantly lower than that in metaphase's endometrial tissues (3.49 ± 1.92 vs 24.69 ± 18.83 , $n = 6$, $P < 0.05$).

HE staining of uterus in early pregnant mice

As shown in Figure 1, there was no significant change in uterine cavity on D1 and no embryo was visible. The uterine cavity was reduced and distorted significantly on D4, and the surrounding glands were increased to prepare for embryo implantation. There was an implanted embryo in the center of uterine cavity on D5, and the cells in the endometrium at the implantation site appeared decidualization. Meanwhile, the structure at the implantation site became loose, indicating that D4-5 was the stage of embryo implantation. The embryo had been completely implanted into the endometrium on D6. At the moment, the cell division in the primary decidual area around the embryo increased, and the nucleus in endometrial cells around the embryo became significantly large. There was obvious uterine cavity and there was no significant change in endometrium at the inter-implantation site on D5 and D6.

Changes of miR-155 in uteri of mice with different days of early pregnancy

As shown in Figure 2, the levels of miR-155 in the uteri of early pregnant mice reduced gradually with the implantation of embryos. The levels of miR-155 in the window period of implantation (D4-5) were significantly lower than that on D1, when the fertilized egg was still in the fallopian tube. Moreover, the expression levels of miR-155 on D6, when the embryo had been successfully implanted into the endometrium and was further developed, were significantly lower than that on D4. In addition, the levels of miR-155 at the implantation site on D5 were significantly lower than that at the inter-implantation site.

Effects of miR-155 agomir injected into uterine horn on mouse embryo implantation

As shown in Figure 3, the amount of embryo implantation in the left uterus injected by mmu-miR-155 agomir was significantly lower than that in the right uterus injected by NC agomir (1.6 ± 0.89 vs 4.4 ± 1.34 , $n = 5$, $P < 0.05$).

Discussion

During the process of embryo implantation, the endometrial cells in the window period could transport, synthesize and secrete some substances, such as adhesion molecules, cytokines, growth factors, lipids and non-coding RNAs, into the uterine cavity to promote the localization and adhesion of embryos [10], in which non-coding RNAs included miRNA. As a regulator of gene expression, miRNA could affect the endometrial angiogenesis, changes of energy metabolism, cell proliferation, establishment of decidualization and inflammatory balance by regulating gene expression and protein synthesis in cells [11], so as to construct good endometrial receptivity. It was reported that DICER and DROSHA, enzymes related to the synthesis of miRNA in the endometrium of window period, in the patients with unexplained primary infertility were significantly lower than that in normal fertile women [12], indicating that they might be closely related to the failure of embryo implantation. Therefore, investigating the role of miRNA in embryo implantation can provide a new theoretical basis for the clinical intervention, diagnosis and treatment of the patients with repeated implantation failure or unexplained early abortion.

In this study, we first collected the endometrial tissues in middle secretory stage from normal women with childbearing age and the decidual tissues of early pregnancy, which represented two different forms of endometrium before and after embryo implantation, and found that the expression levels of miR-155 in decidual tissues after embryo implantation were significantly lower than that in the endometrial tissues before implantation. This was consistent with the result reported by Carlos et al [13], and they found that the expression levels of miR-155 in decidualized human primary endometrial stromal cells induced *in vitro* were down-regulated more than twice. Another study found that miR-155 was closely related to the inflammation of decidual cells [14]. In the next animal experiments of this study, it was found that during the early pregnancy of mice, D4 and D5 were the window period of embryo implantation, and that obvious embryo implantation could be seen on D5 and D6. With embryo implantation, the surrounding endometrium of an embryo began to form decidua, and the expression levels of miR-155 in endometrium decreased. It was reported that the matrix metalloproteinase-2 (MMP-2) and MMP-9 levels in cell culture medium decreased significantly after overexpressing miR-155 [15, 16], indicating that high expression of miR-155 could inhibit the activities of MMP-2 and MMP-9. MMP-2 and MMP-9 participated in the decidualization process of embryo implantation possibly by degrading extracellular matrix [17]. Our results showed that the expression levels of miR-155 decreased gradually with the process of decidualization, which was conducive to embryo implantation. The decrease of miR-155 might increase the enzyme activities of MMP-2 and MMP-9, which would promote the implantation of an embryo.

During embryo implantation, there were a large number of lymphocytes in the uterine decidua, including T cells, uterine NK cells (uNK), macrophages and dendritic cells. These cells participated in the mother-fetal interaction, so as to ensure that the homologous fetal graft was not rejected [18, 19]. Macrophages in the process of pregnancy were divided into two types, pro-inflammatory type-1 (M1) and anti-inflammatory type-2 (M2). Some researchers [20–22] thought that miR-155 participated in the process of M2 macrophage polarization by targetedly regulating the expression of suppressor of cytokine signaling 1 (SOCS1), and that the inhibition of miR-155 could lead to the inhibition of M1 macrophage polarization, promotion of M2 macrophage polarization and reduction of maternal-fetal immune tolerance. In this study, the expression levels of miR-155 at the implantation site of mice on D5 were significantly lower than that at the inter-implantation site, indicating that macrophages at the implantation site had reduced pro-inflammatory activity, which could create a microenvironment of maternal-fetal immune tolerance and would be helpful for the implantation of an embryo. In a clinical study published in 2016, it was found that the expression levels of miR-155 in uNK cells of decidual tissues from patients with recurrent abortion were significantly higher than that from early pregnant women, indicating that miR-155 may be closely related to abortion [23]. Ibrahim et al. [24] treated endometrial stromal cells with lipopolysaccharide (LPS) *in vitro*, and found that abnormal inflammatory activation could cause the high expression of miR-155, and that the pro-inflammatory cytokines such as interleukin 6 and tumor necrosis factor α (TNF α) were also increased significantly. In our study, miR-155 agomir was injected into the uterine horn to simulate the microenvironment state of high expression of miR-155 in the uterus *in vivo*, and it was found that the amount of implanted embryos in the uterus injected by miR-155 agomir was significantly lower than that by NC agomir, indicating that the high expression of miR-155 interfered with the process of embryo implantation, which may be related to the inflammatory activation of maternal-fetal interface caused by the high expression of miR-155. This was consistent with the results of a retrospective clinical study reported by Drissenek et al. [25], who divided the patients into the clinical pregnancy group and non-pregnancy group according to the outcomes of ART, and found that the overexpression of miR-155 in the window period of embryo implantation was closely related to the failure of embryo implantation.

Conclusions

Our study preliminarily investigated the expression pattern of miR-155 in the process of embryo implantation, and found that miR-155 participated in the process of endometrial decidualization during embryo implantation, which might be related to the abnormal activation of immune cells in the process of endometrial decidualization. The results will lay a foundation for further investigating the function and mechanism of miR-155 in the process of embryo implantation, and provide new ideas and directions for the diagnosis and treatment of embryo implantation failure. However, our study had some limits, that is, the size of clinical samples was small. In the future, we will expand the sample size to further validate our results, and investigate the specific molecular regulation mechanism of miR-155 during embryo implantation by *in vivo* and *in vitro* experiments.

Abbreviations

ART

assisted reproductive technology

IVF-ET

in vitro fertilization - embryo transplantation

qRT-PCR

quantitative RT-PCR

IS

implantation site

IIS

inter-implantation site

NC

Normal control

MMP

matrix metalloproteinase

uNK

uterine NK cells

LPS

lipopolysaccharide

TNF α

tumor necrosis factor α

SOCS1

suppressor of cytokine signaling 1

Declarations

Ethics approval and consent to participate

This study was approved by the ethics committee of Zhongda Hospital Affiliated to Southeast University, and the patient's informed consent was obtained.

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YJLi designed the study, obtained the funding and approved the final version to be submitted; YHJ and QM performed the research and acquired data; TS and XZ analyzed the data and wrote the manuscript; JCL analyzed the data and revised the manuscript. All authors read and approved the final manuscript.

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Not applicable.

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Figures

Figure 1

HE staining of uterus in early pregnant mice (×40)

Legends of Figure 1:

D1: Day 1 after coitus. D5: Day 5 after coitus. D6: Day 6 after coitus. IS: Implantation site. IIS: Inter-implantation site. D1: There was no significant change in uterine cavity and no embryo was visible. D4: The uterine cavity was reduced and distorted significantly and the surrounding glands were increased to prepare for embryo implantation. D5 IS: There were implanted embryos in the center of uterine cavity and the cells in the endometrium appeared decidualization. Meanwhile, the structure became loose. D6 IS: The embryo had been completely implanted into the endometrium. At the moment, the cell division in the primary decidual area around the embryo increased, and the nucleus in endometrial cells around the embryo became significantly large. D5 IIS and D6 IIS: There was obvious uterine cavity and there was no significant change in endometrium.

Figure 2

Expression pattern of miR-155 in uteri of early pregnant mice

Legends of Figure 2:

mmu-miR-155: mouse miR-155. D1: Day 1 after coitus. D4: Day 4 after coitus. D5: Day 5 after coitus. D6: Day 6 after coitus. IS: Implantation site. IIS: Inter-implantation site. A: The levels of miR-155 in the uteri of early pregnant mice reduced gradually with the implantation of embryos. B: The expression levels of miR-155 on D6 (after embryo implantation) were significantly lower than that on D4 (before embryo implantation). C: The levels of miR-155 at the implantation site (IS) on D5 were significantly lower than that at the inter-implantation site (IIS). *: $P \leq 0.05$ versus D4 or IIS; **: $P < 0.01$ versus D1; ***: $P < 0.001$ versus D1.

Figure 3

Effects of miR-155 agomir on mouse embryo implantation

Legends of Figure 3:

Left: Injected by mmu-miR-155 agomir: Right: Injected by normal control (NC) agomir. The amount of embryo implantation in the left uterus injected by mmu-miR-155 agomir was significantly lower than that in the right uterus injected by NC agomir.