MicroRNAs in small extracellular vesicles indicate successful embryo implantation during early pregnancy

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Research

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Abstract

Background : Synchronous communication between the developing embryo and the receptive endometrium is required for successful embryo implantation. Assessing of uterine receptivity is important for overcoming the recurrent implantation failure (RIF). Although the potential roles of small extracellular vesicles (sEVs) miRNAs in pregnancy have repeatedly mentioned, the systematic study of sEVs derived from endometrium and its cargoes during the implantation have not yet been reported.

Methods : In this study, sEVs were isolated from mouse uterine cavity on D2 (pre-receptive phase), D4 (receptive phase) and D5 (implantation) of pregnancy using ultracentrifugation and analyzed by transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA). The miRNAs expression profiles of sEVs were identified by RT-qPCR. The datasets analysis of RIF were employed by an integrative bioinformatics analysis. The protein markers of sEVs were examined by western blotting. The effects of miRNAs in embryo implantation were determined by an agomir injection test.

Results : Here we show that the number of multivesicular bodies (MVBs) in the endometrium is increased during the window of implantation (WOI). Meanwhile, the expression of CD63 is mainly located in the luminal and glandular epithelium. sEVs miRNAs profile reveal that miR-34c-5p, miR-210, miR-369-5p, miR-30b and miR-582-5p are enriched during WOI. By further integrating the database analysis results of RIF, we found that miR-34c-5p regulates GAS1 to involve normal process of embryo implantation. It is interesting that miR-34c-5p is down-regulated during implantation but enriched in sEVs. An implication of this is the possibility that sEVs miR-34c-5p could be used to evaluate uterine states.

Conclusion : Our study identified that mouse endometrium release sEVs during the early stage of pregnancy, especially during WOI. We found that miR-34c-5p in sEVs affects embryo implantation by targeting to GAS1. Furthermore, the sEVs miRNAs suggest potential biomarkers for the choose of suitable period for embryo implantation. This study also has a number of important implications for future practice.

Background

Infertility and sub-fertility were major challenges of human reproduction. Although progressions have been made in assisted reproduction technology (ART), especially in the field of embryo transfer technology, to improve the fertility efficiency, the implantation rates remain disappointingly low. The challenge regards the recurrent implantation failure (RIF) causing by the inadequate uterine receptivity and the insufficient communication between the embryo and the uterus[1, 2]. Previous studies have revealed that pregnancy failure mainly occurred during the period of embryo implantation [3]. In most cases, it was very difficult to master the balance between embryo and maternal uterus. Embryo implantation is a vital and complex progress, locates in the early pregnancy in mammals, and be modulated by many factors, including microRNAs, cytokines, growth factors and lipids [4]. Embryo implantation is established only when uterus reach to a status called receptivity and embryos acquire

competency to adhere [5]. Successful implantation requires necessary communications between the embryo and the maternal endometrium [6]. Due to the lack of clear understanding of maternal-fetal crosstalk, the failure ratio of implantation was not improved.

miRNAs are small noncoding RNAs, with 19–22 nucleotides, that regulate the expression of endogenous genes by targeting to 3' untranslated region (3' UTR), resulting the inhibition of protein translation and mRNA degradation [7]. miRNA is one of the most important approaches to adjust the genes expression during embryo implantation [8]. A smart of miRNAs, such as miR-200c [9] and miR-30d [10], took part in the implantation of blastocyst and regulation of uterine receptivity had been verified [11, 12]. Notably, recent studies reveal that miRNAs can be packaged into small extracellular vesicles (sEVs) and delivered to both the embryo and the endometrium, serving as the medium for fetal-maternal communication[13, 14].

Exosomes, a subtype of EVs, were coated by lipid-bilayer with a 40–150 nm diameter. A significant amount of sEVs were considered to be exosomes. sEVs could be released by almost all types of cells [15]. Accumulating evidences suggest that the generation and the release of sEVs are crucial strategies in cellular communication [13]. The cargos of sEVs affected the biological processes of recipient cells during physiological and pathological states [16]. There is growing evidence suggested the potential role for sEVs in early pregnancy [17–19]. Previous researches had revealed that endometrium secrete viscous fluid into uterine cavity throughout the menstrual cycle [17, 20]. sEVs have been verified to exist in the uterine fluid-the viscous fluid secreted by the endometrium. The cargos of UF secretions vary with the remodeling of the endometrium throughout the menstrual cycle [21, 22]. More important, a vast evidences indicated that sEVs played potential roles in early diagnosis for diseases [23, 24], as well as embryo implantation [25]. Hence, sEVs could provide great values for evaluating the endometrial status in the specific phase, such as the WOI. Unfortunately, current studies couldn't provide a well understanding of sEVs for uterine physiological biopsy during early pregnancy.

In this study, the morphology of endometrium was examined during the pre-implantation, implantation and post-implantation period in a mouse model. A comprehensive investigation of sEVs derived from endometrium in these stages of early pregnancy was performed. We detected the miRNAs profile in sEVs and several sEVs miRNAs were identified to be associated with successful implantation. The main purpose of our study is to identify sEVs miRNAs derived from endometrium during early pregnancy and propose a novel marker for evaluation of endometrial physiology. These sEVs miRNAs also could provide a new idea for clinic therapy and monitor of infertility.

Materials And Methods

Cell culture

Ishikawa and HEC-1-A cell lines were purchased from cell bank of Chinese Academy of Science (Shanghai, China). Ishikawa cells were cultured in DMEM (Gibco) culture medium supplemented with

10% FBS. HEC-1-A cell lines were cultured in McCoy's 5A (SIGMA) supplemented with 10% FBS. For primary endometrium cells (pECs) isolation, the mice on D4 of pregnancy were used. In brief, the uterus were removed into PBS supplemented with 2% penicillin-streptomycin solution. Then fat tissues and blood were carefully removed. Uterine tissues were cut into small pieces and digested with collagenase I. FBS was used to end digestion. The dispersed tissues were then filtered with a 100 μ m cell strainer, and the supernatants were centrifuged at 1000 rpm for 5 min. pECs were cultured in DMEM (Gibco) culture medium supplemented with 10% FBS.

sEV Isolation

Adult female mice (ICR, 8weeks) were mated with fertile males randomly. Mice were housed in a specific pathogen-free facility, and the experimental protocols were approved by the College of Animal Science, Zhejiang University. The uterus of mice on D2, D4 and D5 of pregnancy were used for isolation of sEV. In brief, mice were perfused through the heart at the left ventricle with cold PBS at a rate of 5 ml/2 min to remove the blood in tissues. The cervix was ligated carefully to avoid uterine fluid lost, and the excess fat and mucous tissues were removed from uterus. Uterine horn was washed with 1 ml cold PBS in each side. The fluid were then collected. Next, endometrium was scraped from uterus and digested with 0.25% Trypsin solution for 30 min. Exosome free FBS was used to terminate the effect of Trypsin. Tissues debris were removed by a ephermal centrifugation. Then the supernatants were mixed with uterine fluid. The liquid was centrifuged at 600 g for 5 min to remove dead cells, 1200 g for 15 min to remove debris and 10000 g for 30 min to remove microvesicles. The supernatants were filtered through a 0.22 μ m Millipore. Clarified fluid was centrifuged using a type 70 Ti rotor (Beckman) at 120000 g for 75 min at twice. The pelleted sEVs were suspended in PBS for further usages.

Immunohistochemistry

Uterus were collected from mice on D2, D4 and D5 of pregnancy. The uteri were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned (5 μ m). After deparaffinized, the sections were incubated in boiling 10 mM citrate buffer (pH 6.0) for antigen retrieval. Then the sections were blocked with 10% FBS and incubated with antibody CD63(Abcam, Cat.No. ab217345) (1:100) overnight at 4 °C. Sections were washed in PBS and incubated with biotinylated secondary antibody for 2 h at room temperature. The image was captured by NIS Elements software using light microscopy (NiKon).

sEVs Tracking Analysis

sEVs were incubated with Dil dye for 15 min at room temperature, and 5% BSA was used to avoid over-stain. The labeled sEVs were diluted with PBS and centrifugated at 120000 g for 75 min. Finally, the pellets were resuspended in PBS. Then the labeled sEVs were added to pECs cell culture supplemented with 10% exosome-free FBS and incubated for 12 hours. Cells were washed and fixed with 4%

paraformaldehyde. Following the cells were treated with 0.1% Triton X-100 for 30 min and incubated with a FITC-conjugated phalloidin (Solarbio, Cat.No. CA1620). DAPI was used to mark nucleus. The cells were visualized by a confocal microscopy (ZEISS, Germany) and captured or processed using ZEN 100 software. Exosomes-depleted FBS was prepared by ultracentrifugation overnight at 100,000 g, 4°C.

Transmission Electron Microscopy

For identification of sEV, sEVs were placed onto the formvar carbon-coated copper grids at RT for 1 min. The excess suspension was removed using a filter paper. Then sEVs were rinsed double distilled water 3 thrice. Following sEVs were stained using 2% uranyl acetate at RT for 1 min. The grids were then dried in the air. Image was observed with a Tecnaig2 Spirit120KV transmission electron microscope operating at 120 kV (Thermo FEI).

For observation of MVBs in endometrial tissues, a standard TEM method was used. Briefly, fresh tissues were fixed with 2.5% glutaraldehyde overnight at 4°C and washed with PBS. Tissues were fixed again with 1% osmic acid, stained using 2% uranyl acetate, dehydrated in gradient alcohol and embedded in epoxy resin. Ultrathin sections were transferred onto carbon-coated copper grids and examined using a Tecnaig2 Spirit120KV transmission electron microscope operating at 120 kV (Thermo FEI).

Scanning Electron Microscopy (SEM)

For characterization of the morphological structures of luminal epithelium in early pregnancy, a SEM process was performed. In brief, endometrium tissues were fixed with 2.5% glutaraldehyde overnight at 4°C and rinsed in water. Then the tissues were fixed again with 1% osmic acid, stained with 2% uranyl acetate and dehydrated in gradient alcohol. Following the samples were dried out of liquid CO₂ at critical point and coated with platinum. The tissues were scanned with a Nova Nano 450 scanning electron microscopy and images were captured (Thermo FEI).

Nanoparticle Tracking Analysis

NTA was used to determine the sizes distribution and concentration of sEVs. sEVs were resuspended and diluted with PBS for analysis using a ZetaView PMX 100 (Particle Metrix, Meerbusch, Germany). Particle movement was analyzed using NTA software (ZetaView 8.02.28). For each group, at least three independent experiments were performed.

Western Blot Analysis

The concentration of proteins were measured by a BCA protein kit (Beyotime, Cat. No. P0010S). Total proteins were dissolved using 6–12% SDS-PAGE and transferred to the polyvinylidene difluoride

membranes (Millipore). The membranes were then blocked with QuickBlock™ western buffer (Beyotime, Cat. No. P0231) for 20 min at room temperature and incubated with primary antibody including CD63 (Abcam, Cat.No. ab217345), CD9 (Diagbio, Cat.No. db919), Alix (Cat.No. db3856), HSP70 (Cat.No. db2396), Calnexin (ABclonal, Cat.No. A0803), GAPDH (Diagbio, Cat.No. db106) and GAS1(absin, Cat.No. abs141177) at 4°C overnight. Following the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Abclonal, 1:3000) at room temperature for 2 h. The blots were detected using BeyoECL Plus (Beyotime, Cat.No. P0018S).

mRNA Datasets Analysis

The Gene Expression Omnibus (GEO) (<https://www.ncbi.nlm.nih.gov/geo/>) is a publish database for storing high-throughput gene expression datasets. Through the retrieval of GEO database by keywords (endometrial receptivity, WOI, recurrent implantation failure, embryonic implantation and miRNA), four gene expressed chips associated with RIF (GEO Database: GSE111974, GSE103465, GSE26787, and GSE92324) were retrieved. The expressed data of microarrays were normalized before analysis. A limma package was employed in R basic for differentially expressed genes (DEGs) analysis, and $|\log FC| > 1.0$ and $P \text{ value} < 0.05$ were set as the threshold. Then the DEGs were screened and draw custom Venn diagrams (<https://bioinformatics.psb.ugent.be/webtools/Venn/>) was used to compare the DEGs in four datasets. Five miRNA-mRNA relation prediction databases (Targetscan, miRwalk, miRDIP, miRSearch and miRtarBase) were applied to predict the target genes of miRNAs.

miRNA Agomir Injection

miR-34c-5p agomirs were purchased from the GenePharma Company (Shanghai, China). Agomirs were diluted to the concentration 20D/5 ul. A surgical operation was performed for the mouse on D3 of pregnancy. 5 ul miR-34c-5p agomir was injected into one side of the uterine horn, and 5 ul DEPC water as a control was injected into the other side of the uterine horn. The number of implantation sites were checked on D7.

RNA Extraction And Qrt-pcr

Total RNAs were extracted from the endometrium tissues using Trizol reagent (TIANGEN, Cat. No. DP421), according to the manufacturer's protocols. Antisense strand of RNAs were synthesized by the first strand cDNA synthesis kit (TIANGEN, Cat. No. KR118). The relative expression of mRNA level was detected using SuperReal PreMix Color (SYBR Green) qRT-PCR kit (TIANGEN, Cat. No. FP215). miRNAs were isolated from endometrial tissues, cells and sEVs using miRNeasy Mini Kit (QIAGEN, Cat. No. 217184). Then RNA was eluted in RNase-free water and reverse-transcribed to cDNA following the kit protocol (TIANGEN, Cat. No. KR211). cDNA samples were run using miRcute Plus miRNA qPCR Kit (SYBR Green) (TIANGEN, Cat. No. FP411). GAPDH, U6 or cel-miR-39 was used as a control. Relative miRNA and

mRNA expression levels were assessed by the $2^{-\Delta\Delta Ct}$ method. All samples were tested in triplicate at least. The information of primers as follows. GAPDH: forward, ACAACTTTGGTATCGTGGAAGG; reverse, GCCATCACGCCACAGTTTC. cel-miR-39: TCACCGGGTGTAATCAGCTTG. U6: AACGAGAAGCGAACCAAAAAA. Human GAS1: forward, ATGCCGCACCGTCATTGAG ; reverse, TCATCGTAGTAGTCGTCCAGG. Mouse GAS1: forward, CCATCTGCGAATCGGTCAAAG ; reverse, GCTCGTCGTCATATTCTTCGTC.

Statistical analysis

All experiments in this study were performed three independent times at least. The values were reported as mean $\pm$ s.e.m. Student's t-test was employed to compare the significance in two group. One-way analysis of variance (ANOVA) was performed to compare multiple (> 2) means. Plots were used GraphPad Prism 6.0 or R basic. P-value ≤ 0.05 was considered to be significant. As follows: ** P-values ≤ 0.01 , * P-values ≤ 0.05 and ns P-values ≥ 0.05 .

Results

Extracellular vesicles are present and secreted by endometrium

The cell-surface of the uterine epithelial cells has been through drastic morphological changes during the peri-implantation stage. Most apparent changes are witnessed in the microvilli on the apical membrane of the cells [26]. SEM results revealed that the microvilli on the luminal epithelium became shorter during the WOI (D4 and D5) compared with the pre-implantation stage (D2) (Figure S1a). Pinopode, a flatten and smooth protrusion that presences on the surface of the endometrium during implantation, serving as a receptive marker [27], were also detected on D4 of pregnancy(Figure S1a), indicating that the uterus had entered the receptive stage. Surprisingly, we also observed some vesicles on the surface of endometrium similar to sEVs (Figure S1a, red arrows indicated). Current studies consider that sEVs originated from early endosome and are packaged in the multivesicular bodies (MVBs) [28]. In addition, during the pregnancy, a majority of molecular components, including EVs were released into the uterine cavity to regulate the physiological processes [29, 30]. Hence, in order to gain further insights into the release of sEVs by the endometrium in early pregnancy, TEM was performed to investigate MVBs in endometrium on D2, D4 and D5 of pregnancy. The TEM results suggested that MVBs containing the typical intraluminal vesicles (ILVs) were presented in endometrium during implantation (Fig. 1a). These MVBs and ILVs had distinct lipid bilayer and the size of ILVs were similar to the sEVs. In those three different physiological stages of endometrium during early pregnancy, no significant difference was identified on the size and number of MVBs (Fig. 1c, d). However, the number of ILVs per MVBs were increased during WOI when compared to the pre- and post-implantation stage (Fig. 1b). An interesting phenomenon was also noticed, EVs were observed in the extracellular space (Figure S1b, c). These data supported that endometrium secreted sEVs.

Characterization Of Endometrium Derived sEVs

To confirm the secretion of sEVs by the endometrium, CD63, a well-documented marker of the sEVs [31], was examined. CD63 was also confirmed to be important for embryo implantation [32]. Immunohistochemistry results indicates that CD63 was mainly expressed in the luminal and glandular and epithelium of endometrium (Fig. 2). Notably, its expression was low in the pre-implantation stage and increased significantly during the implantation stage. Given that CD63 is enriched in the sEVs, which had been shown to present in the uterine cavity, we thus suspected a vivid secretion activity may happen during the implantation stage. In this study, a mixture of uterine fluid and scraped/trypsin treated endometrium were collected during implantation. We used a standard protocol to isolate sEV from endometrium (Fig. 3a). We performed TEM to investigate the morphology of particles isolated from endometrium. The results showed that these oval or bowl-shaped particles (Fig. 3b) presented a typical morphology of what have been generally described for sEVs [15, 16, 33]. Western blot analysis showed the enrichment of the sEV markers such as CD63, CD9, Alix, and HSP70 (Fig. 3c). As a negative control, endoplasmic reticulum protein Calnexin was absent in our sEVs samples. To confirm the purification of sEVs isolated by our methods, CD63, Alix and Calnexin were also detected in the endometrium tissues, the 500 g pellets, the 1200 g pellets and the 10000 g pellets. These results suggested that we purified the sEVs from the endometrium without cell debris population (Fig. 3c). Consistent with the TEM and the western blotting results, NTA analysis demonstrated that the sizes of sEVs were enriched with range between 40 to 200 nm (Fig. 3d). The mean size of sEVs in each group was 133.9 nm, 137 nm, and 131.1 nm, respectively. Next, we questioned whether sEVs could be uptaken by the endometrial cells. sEVs from endometrium play a vital role in regulation of endometrial physiological environment. Therefore, primary endometrium cells (pECs) were separated and incubated with labeled sEVs derived from endometrium on D2, D4 and D5 of pregnancy. The red fluorescence signal of confocal images showed that labeled sEVs were endocytosed by pECs (Fig. 3e). On the contrary, no fluorescence signal was detected in the negative control. Thus, our data indicated that endometrium released sEVs into uterine lumen and sEVs could be delivered to endometrial cells in the period of implantation during early pregnancy.

Endometrium Derived sEVs Carry Receptive miRNAs

The establishment of uterine receptivity is a prerequisite for successful embryo implantation. MiRNAs have been reported to participate in the regulation of uterine receptivity. Dynamic variations of MiRNAs had been detected during early pregnancy, suggesting a vivid miRNA activity [11]. To investigate the presence of miRNAs in the sEVs released by endometrium, the expression profile of 23 miRNAs were selected to be examined in sEVs. These miRNAs were related to the regulation of uterus receptivity [10–12]. sEVs derived from endometrium on D2, D4 and D5 of pregnancy expressed all the selected miRNAs (Fig. 4a). Among the identified miRNAs, most miRNAs were down regulated in the sEVs during the WOI and the post-implantation stage. However, miR-34c-5p, miR-210, miR-369-5p, miR-30b and miR-582-5p were markedly increased in sEVs during WOI when compared to pre-implantation. Most importantly,

studies revealed that miR-34c-5p, miR-210 and miR-30b were packaged in sEVs and released by multi-types cells [34–36]. The expressed level of miR-34c-5p, miR-210 and miR-30b were significantly increased during implantation. In addition, in an attempt to reveal the potential function of the sEVs derived miRNAs up/down regulated during implantation, we investigated that the mRNAs were potentially targeted by miRNAs in sEVs. The prediction analysis of bioinformatics revealed that 2987 mRNAs were predicted targets of these miRNAs, which were mainly enriched the PI3K-Akt signaling pathway, MAPK signaling pathway, Focal adhesion, cell-cell adhesion and extracellular exosome (Fig. 4(b,c,d)).

miR-34c-5p targets to GAS1 in RIF endometrium

Initially, four microarrays datasets about RIF (GEO Database: GSE111974, GSE103465, GSE26787, and GSE92324) were used in this study. R basic was employed to screen DEGs between normal implanted and RIF endometrial tissues (with conditions: $|\log FC| > 1.0$ and $P \text{ value} < 0.05$). The DEGs of each dataset were plotted in Venn diagram (Fig. 5a). GAS1 (growth arrest specific 1) was determined to be the exclusive intersection gene. The detail results of top 400 DEGs in each chip were showed in Table S1. The genes expression profile of top60 DEGs from GSE103465 and GSE111974 were showed in heatmaps, respectively (Fig. 5(b, c)). The expression of GAS1 was observed to be lower in the RIF endometrium than the normal endometrial tissues of women (Fig. 5d). Furthermore, we predicted the miRNA regulating GAS1 through five miRNA-mRNA relation prediction databases (Targetscan, miRwalk, miRDIP, miRSearch and miRtarBase). The detail information of predicted miRNAs (only show top28 which have high scores) in each database were displayed in Table S2. In our results, two miRNAs, has-miR-34c-5p and has-miR-34a-5p, were found by comparison of prediction results (Fig. 5e). The results indicated that these miRNAs were highly likely to regulate GAS1 in endometrium of women. In order to further identify the regulated relationship between miR-34c-5p and GAS1, GSE71332 (a miRNAs expression chip of GEO database) was screened by a bioinformatics approach. Only miR-34c-5p showed significantly low expression in the normal fertile endometrial tissues (Fig. 5f). The specific expression data of GAS1 and miR-34c-5p were depicted in Table S3. Based on these results, we supposed that GAS1 had a positive function during embryo implantation. On the contrary, miR-34c-5p play a negative role in this process, probably through regulating GAS1 expression. Hence, two human endometrial cell lines, Ishikawa and HEC-1-A, were used to verify the hypothesis. Ishikawa cells present the feature for receptive endometrial cells as they are poorly polarized, serving as a good model for receptivity-associated research, while HEC-1-A cells display low adhesive properties for embryo adhesion, which makes them non-receptive endometrial cell lines [37]. The results showed that GAS1 was highly expressed in Ishikawa cells when compared with HEC-1-A cells (Fig. 5g). However, the expression pattern of miR-34c-5p was opposite in two cell lines (Fig. 5h). These results suggested that GAS1 was likely to involve the embryo implantation and regulated by miR-34c-5p in the endometrium.

miR-34c-5p is poorly expressed during embryo implantation

The mature sequence of miR-34c-5p and its binding area in 3'UTR of GAS1 were conserved in human and mouse (Fig. 6a). In order to further verify the relationship between miR-34c-5p and GAS1 in mouse model, the expression of GAS1 and miR-34c-5p were determined in the endometrium during early pregnancy through the application of RT-qPCR (Fig. 6b and c), respectively. In our results, GAS1 was highly expressed during the WOI (Fig. 6b). However, GAS1 was poorly expressed during the pre- and post-implantation period, indicating the significant roles of GAS1 may have during embryo implantation. On the contrary, miR-34c-5p was down-regulated during WOI when compared to pre- and post-implantation period (Fig. 6c). We also detected the protein level of GAS1 during early pregnancy (Fig. 6d). The results were consistent with the mRNA expression. Next, an injection experiment was performed to further identify the regulation of GAS1 by miR-34c-5p *in vivo*. Because of the expression of miR-34c-5p was poorly in D2, D3 and D4, agomir of miR-34c-5p was injected into the uterus of pregnant mouse in D2. After 48 h of injection, the endometrium in D4 were collected to detect the expression level of GAS1 (Fig. 6e). Interestingly, GAS1 was actually down-regulated after injection of miR-34c-5p agomir when compared to control group in mouse model (Fig. 6g). Thus, miR-34c-5p regulated GAS1 *in vivo* during embryo implantation. Meanwhile, we investigated the impact of miR-34c-5p in number of implanted embryos. miR-34c-5p agomir was injected into uterus on D3 and the number of implanted embryos were examined four days later (Fig. 6f). We found that there was a significantly lower number of embryos in side of agomir injected horn than the other side of control (Fig. 6h). Therefore, we suggested that reinforcement of mmu-miR-34c-5p during WOI triggers a risk of embryo loss (Fig. 6i). Collectively, these data confirmed a critical role of miR-34c-5p in maintaining a normal embryo implantation during early pregnancy by targeting GAS1.

Discussion

In this study, sEVs were derived from a mixture of uterine fluid and scraped/trypsin treated endometrium. Uterine secretions were complex and not well characterized, but those components present in the uterine lumen of female mouse or human were likely derived primarily from the endometrium. Hence, the specific source of EVs in extracellular environment could not be well determined. According to the MISEV2018 guideline [38], we named these vesicles isolated in our study with 40–200 nm diameter using "sEVs" instead of "exosomes". In this study, the overall experiments were performed with MISEV guidelines, including characterization of sEVs with respect to quantitation, morphology, size and protein markers. In fact, sEVs had been paid more and more attention to non-invasive diagnostic tool in many diseases [23, 39].

Recently, sEVs have been widely recognized as a novel mechanism for signal communication between cells. Non-coding RNAs and proteins were packaged in sEVs and transferred into specific recipient cells to affect their biological behaviors. As an important mediator of dialogue, an increasing interest of sEVs miRNAs have been centred, [7]. Implantation involves intricate communication between embryos and the maternal endometrium, and sEVs were considered to potentially involve this process [30, 40]. There were also direct evidences demonstrated that the release and uptake of sEVs between the embryo and endometrial cells were bidirectional [41]. EVs derived from the endometrial cells or the inner cell mass of

the blastocyst could regulate the ability (migration and invasion) of embryonic trophoblast cells to establish successful implantation [42]. However, many researches kept a watchful eye on cell communication via EVs in vitro, less studies focused on the sEVs in physiological statuses of uterus in vivo during implantation. In this study, A comprehensive and sequential study of sEVs derived from endometrium during the implantation was conducted. We reported the miRNAs in sEVs associated with successful embryo implantation.

The changes of endometrial morphology are related to normal uterine physiology during the menstrual cycle of women. In early pregnancy, the remodeling uterus purpose to provide a environment for embryo implantation including epithelial-mesenchymal transition [43] and decidualization [44]. Here, An initial objective of the study was to identify the physiological states of implantation. The results of SEM indicated that the uteri were collected on D2, D4 and D5 of pregnancy which refers to pre-implantation, implantation and post-implantation period, respectively. Studies have demonstrated that uterus secreted abundant factors into the uterine cavity to nurture embryo during early pregnancy [45]. In this study, vesicles similar to sEVs were observed to be located in the endometrial surface. Although there are many studies on release of endometrial cell lines [18, 20, 25, 46], no studies have provided the evidence of sEVs derived from the endometrium during early pregnancy in vivo. sEVs were mainly originated from MVBs and ILVs, and were released when MVBs fused with plasm membrane [47]. The results of this study showed that MVBs presented in endometrium during implantation and the number of ILVs per MVBs were significantly increased during WOI. In addition, CD63 was also observed mainly located in the uterine luminal and glandular epithelium. These results support that endometrium potentially secrete sEVs during early pregnancy in mouse.

Previous studies had isolated exosomes/EVs from UF [17], but there lack the study containing whole stages of early pregnancy from early to late secretory. sEVs were presented in UF and endometrial tissues. In view of this, we collected UF and endometrial tissues from the period of pre-implantation, WOI and post-implantation. sEVs were isolated and purified. sEVs derived from endometrium contributed to the regulation of appropriate uterine physiological states for implantation. The organ- and tissue-secreted sEVs carried with specific cargos in different stages of physiology [48]. sEVs are thought to be fused with plasm membrane of recipient cells and the contents of sEVs were transferred into target cells. In our results, these sEVs could be delivered to the endometrial cells. In addition, miRNAs were the main source of circulating miRNAs and sEVs enriched miRNAs selectively with the development of diseases [49]. Studies have reveled that the diagnostic of miRNAs in sEVs increased with cancer development [50]. Previous study of human miRNAs profiling revealed that maternal miRNAs were were released into the UF during the window of implantation and were packaged in exosomes [20]. Unlike previous studies, we directly analyzed the expression profiles of miRNAs in sEVs, rather than in UF, which avoided interference of other components in the UF. These sEVs miRNAs, which associated with uterine receptivity and regulated cell migration, cell invasion, cell adherence and proliferation, were mainly existed in UF. This provided a new sight way for us to estimate the states of uterus. Infertility is a problem of reproduction, and in vitro fertilization is costly and has a relatively low success rate, in general below 30% [3]. One of the causes of failure during in vitro fertilization and embryo transplantation is the inadequate of uterine

receptivity to allow embryo implantation [51]. More and more studies have proved that sEVs miRNAs could serve as a potential biomarker for diagnosis and treatment [23, 52, 53]. In our study, miR-34c-5p, miR-210 and miR-30b were observed to be specially expressed in sEVs during implantation. We proposed that these sEVs miRNAs might indicated the suitable uterine environment for embryo implantation.

The most important purpose of our study is to provide a biomarker for uterine states detection. Embryo transplantation needed a suitable time when uterus could accept it, and the uterine states and environment of RIF women could be regulated and improved by artificial methods. Although the new technology has achieved some consequents in reproductive, RIF is still an enormous problem. Our results of database analysis showed that GAS1 associated with RIF. GAS1 played a role in embryonic development and human diseases and involved stem cell renewal and cancer growth [54], but the roles of GAS1 in embryo implantation have not been reported. Here we showed that GAS1 was more significant expressed in receptive endometrium than non-receptive endometrium during early pregnancy, indicating the function of GAS1 during this process. Here we also identified that miR-34c-5p targeted to GAS1 in endometrium during embryo implantation. The suppressing of miR-34c-5p was necessary for embryo implantation during WOI. A series of studies have reported that miR-34c-5p involved the process of endometrial receptivity and inflammation [55, 56]. An interesting study revealed that miR-34c-5p inhibited exosome release by targeting RAB27B, a molecule that promotes exosome shedding [34]. miR-34c-5p was down-regulated during WOI, following the secretion of sEVs was increased, possibly because of the regulation between miR-34c-5p and RAB27B. On the other hand, miR-34c-5p was enriched in sEVs in UF during WOI. It is likely a castoff of endometrium, but the sEVs miR-34c-5p could potentially reflect the receptivity of endometrium. This benefits assisted reproductive technology and embryo transplantation, as well as the therapy for RIF. We could easily collect the uterine secretions with a non-invasion approach to determine the optimal period for improving embryo implantation through the detection of sEVs miRNAs.

Conclusion

Our data demonstrates that sEVs are released by the endometrium in mouse early pregnancy, and the miRNAs profile of the secreted sEVs varies with the physiological states of the uterus. Furthermore, miR-34c-5p was confirmed to be important for the embryo implantation. MiR-34c-5p was also observed in sEVs and the expression of miR-34c-5p increased during the early stage of pregnancy. In summary, our findings open up a new avenue, which proposed a new molecule for facilitating successful implantation. In addition, the miRNAs in sEVs provide a positive strategy for therapy of RIF.

Abbreviations

sEVs:small extracellular vesicles; RIF:recurrent implantation failure; ART:assisted reproduction technology; DMEM:Dulbecco's modified Eagle medium; FBS:fetal bovine serum; miRNAs:microRNAs; pECs:primary endometrium cells; TEM:transmission electron microscopy; NTA:Nanoparticle Tracking

Analysis; SEM:Scanning electron microscopy; ILVs:intraluminal vesicles; WOI>window of implantation; MVBs:multivesicular bodies.

Declarations

Acknowledgments

Not applicable

Authors' contributions

Q.T. , Z.G.W. and S.S. designed the experiments and analyzed the data. Q.T. and S.S. wrote the paper. Q.T. , S.S. and J.J.L. conducted the experiments. X.W.Z. and D.R.C. helped with analyzing the data. All authors read and approved the final manuscript.

Authors' information

Not applicable

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Availability of data and materials

A part of data that support the findings of this study are available in GEO at <https://www.ncbi.nlm.nih.gov/geo/>. These data were derived from the following resources available in the public domain: GSE111974, GSE103465, GSE26787, and GSE92324.

Other data generated in this study are included in the manuscript.

Ethics approval and consent to participate

The animal experimental protocols were approved by the College of Animal Science, Zhejiang University.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 2

MVBs are present in mouse endometrium cells during early pregnancy. a Representative electron microscopic images of endometrium tissues on D2, D4 and D5 of pregnancy, respectively. Red arrows indicated MVBs including classic ILVs. b The number of ILVs per MVBs, c size of MVBs, d number of MVBs per cell in different stages. MVBs counted only containing typical ILVs. **P<0.01. ns: no significant.

Figure 4

Immunohistochemical staining of CD63 in the mouse endometrium during the pre-implantation (D2), WOI (D4) and post-implantation (D5) stage. LE: luminal epithelium. GE: glandular epithelium. IS: implantation site. IIS: inter implantation site.

Figure 5

Identification of sEVs derived from endometrium. a Schematic diagram showed the strategy of isolation and purification of sEVs derived from mouse endometrium during early pregnancy. b TEM images showed sEVs derived from mouse endometrium on D2, D4 and D5 of pregnancy, respectively.

Magnification: up, 18500. Down, 68000. c Western blotting analysis of sEV protein markers (CD63, Alix, CD9, and HSP70). Calnexin used as a negative control. d. NTA suggested size distribution and concentration of sEVs. e Confocal microscopic images showed uptake of labeled sEVs by pECs. Nuclei stained by DAPI in blue, sEVs stained by Dil in red and actin stained by FITC-conjugated phalloidin in green. (Bar 20µm)

Figure 8

Endometrium derived sEVs carry miRNAs which associated with uterine receptivity. a Heatmap showed the expression profiles of miRNAs in sEVs derived from endometrium on D2, D4 and D5 of pregnancy. (b-d) GO/KEGG terms of genes were potential targeted by miRNAs in sEVs. b A bubble plot of KEGG pathways enriched. c A bubble plot of GOs (Biological Process) enriched. d A bubble plot of GOs (Molecular function and Cellular components) enriched.

Figure 10

GAS1 was targeted by miR-34c-5p. a The Venn diagram of co-expressed DEGs in Four GEO databases (GSE26787, GSE111974, GSE103465 and GSE92324), only one gene GAS1 was observed. b and c Heat map shown the top 60 differentially expressed genes in GSE103465 and GSE111974, respectively. d The expression of GAS1 in four gene microarrays. e The comparison of target miRNA of GAS1 predicted by TargetScan, miRSearch, miRTarBase, miRWalk, and miRDIP. f The expression of hsa-miR-34c-5p in miRNA

expression chip GEO: GSE71332 of RIF. g The mRNA level of GAS1 in Ishikawa and HEC-1-A cell lines. h The expression of miR-34c-5p in Ishikawa and HEC-1-A cell lines. * P<0.05, ***P<0.001.

Figure 12

miR-34c-5p regulated embryo implantation by targeting to GAS1 in endometrium during early pregnancy. a The regulating relationship between GAS1 and miR-34c-5p, the mature sequence of miR-34c-5p in mouse and human were predicted by Targetscan. b and c The relative expression of GAS1 and miR-34c-5p in mouse endometrium detected by qRT-PCR during early pregnancy. d The protein expression level of GAS1 in mouse endometrium. e The schematic diagram shown that miR-34c-5p regulated the expression of GAS1 in endometrium in vivo. f The schematic diagram shown that miR-34c-5p regulated the successful embryo implantation. g The expression of GAS1 in D4 mouse endometrium after injected miR-

34c-5p agomir in D2 of pregnancy. h and i Effect of in vivo injection of miR-34c-5p agomir on the number of embryos. bar=1cm.

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