Placental trophoblast cell-derived exosomal microRNA-1290 promotes the interaction between endometrium and embryo by targeting LHX6

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Communication between the maternal uterus and the embryo is vital for a successful pregnancy. Exosomes, subtypes of extracellular vesicles comprising many bioactive factors, regulate the early stages of pregnancy, specifically during embryo implantation. Nevertheless, the mechanism by which exosomal microRNAs (miRNAs) derived from placental trophoblasts regulate embryo implantation remains elusive. We isolated and identified exosomes derived from placental trophoblast cells (HTR8/SVneo). Subsequently, we evaluated the loading miRNA in exosomes by small RNA sequencing. Consequently, we showed that trophoblast cell-derived exosomes could transfer to endometrial epithelial cells. Besides, these exosomes promoted the epithelial-mesenchymal transition (EMT) as well as migration of endometrial cells and were implicated in the regulation of inflammation. Further, the specific miRNAs were screened in exosomes, and as a result, miRNA (miR)-1290 was enriched specifically in exosomes. miR-1290 promoted the expression of inflammatory factors (interleukin [IL]-6 and IL-8) and migration of endometrial epithelial cells. In addition, exosomal miR-1290 promoted angiogenesis *in vitro*. More importantly, by targeting LHX6, trophoblast HTR8/SVneo cell-derived exosomal miR-1290 promoted the EMT process of endometrial epithelial cell HEC-1-A. Altogether, our findings provide novel insights into the mechanism of trophoblast cell-derived exosomes during embryo implantation.

INTRODUCTION

Exosomes are substances released by cells with membrane structures; they mediate cell communication.[1] Based on different sizes, biological characteristics, and origins, extracellular vesicles (EVs) can be divided into two main subsets including exosomes (size from 30 to 200 nm) and microvesicles (200−1,000 nm). Studies on exosomes have matured due to their diversity of proteins and nucleic acids as cargos.[2] Noncoding RNAs, including microRNAs (miRNAs), cicrRNAs, and lnRNAs, are specifically enriched in exosomes.[3] Moreover, previous studies demonstrated that exosomes are implicated in numerous physiological and pathological processes, including tumor metabolism, immune regulation, embryo implantation, etc.[4] Notably, nearly all types of eukaryotic cells

secrete exosomes, isolated from body fluids, which potentially serve as biological indicators.[5] These exosomes, derived from host cells, transfer their contents to the recipient cells, where they regulate gene expression and signal activity causing phenotypic and functional changes in downstream cells. Numerous studies focus on the function of exosomes in pathological processes, confirming that exosomes promote cell migration, invasion, and metastasis.[6] In recent years, accumulating evidence indicates that exosomes also regulate normal physiological mechanisms, including immunity[7] and reproduction.[8]

During the early pregnancy period of mammals, the interaction between the uterus and embryo is crucial for successful embryo implantation and development.[9] Morphological and physiological changes occurring in the endometrium, including epithelial-mesenchymal transition (EMT) and decidualization, are mediated by ovarian steroids, estrogen, and progesterone. During the window of implantation,[10] the endometrium reaches a specific state called the receptive state. This is where the endometrium accepts the adhesion and penetration of the embryo. The endometrium also secretes several factors that regulate and nourish the embryo,[11] including miRNAs, proteins, and lipids. At the same time, the embryo differentiates into two major types of cells, i.e., the inner cell mass and embryonic trophoblast cells.[9] The former remains totipotency, whereas the latter continue growing, finally attaching themselves to the uterine wall. Eventually, trophoblast cells invade the uterus and develop into the placenta, forming a stable connection with the mother.[12] Recent studies reveal that the uterine fluid secreted by the uterus contains several EVs with nucleic acids[13] and proteins.[14] These vesicles deliver bioactive substances to the embryo, modulating intra- and inter-cellular communications. Additionally, the communication between the uterus and embryo mediated by the exosomes is bidirectional.[15,16] Nevertheless, most studies
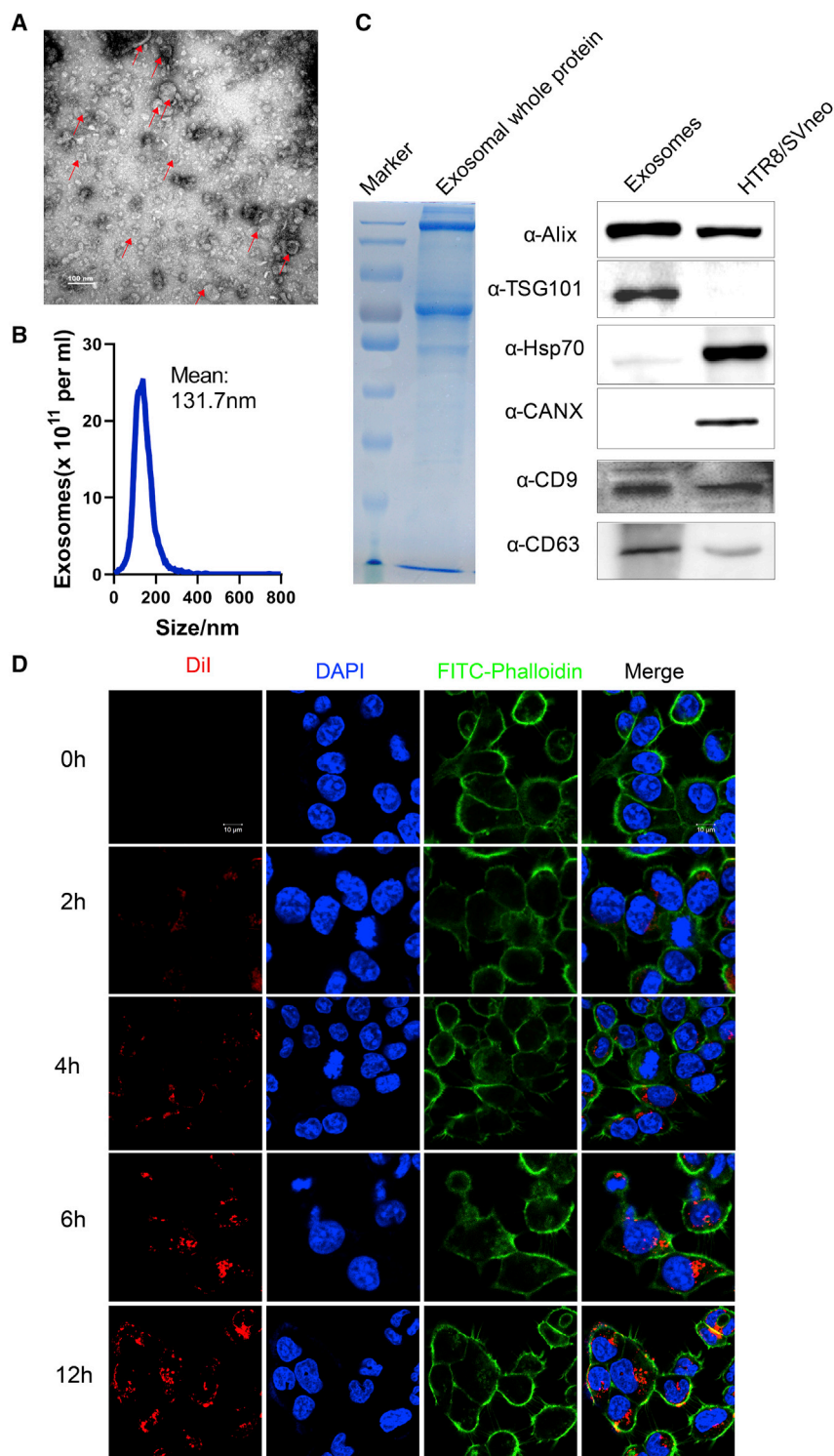
Figure 1. Exosomes isolated from trophoblast cells and absorbed by endometrial cells

(A) The morphology of exosomes was charactered by TEM after negative staining. Bar, 100 nm. (B) The size distribution of exosomes was detected using NTA. (C) Western blot analysis showing exosome markers CD63, CD9, Alix, HSP70, and TSG101 and CANX as a negative control (NC). Coomassie brilliant blue staining showed the protein-loading amount and protein profile of exosomes. (D) The confocal microscopic images showed uptake of exosomes by endometrial cells in a time-dependent manner. Nuclei stained by DAPI in blue and exosomes stained by DiI in red. Bar, 10 μm.

miRNAs are small non-coding RNAs that promote gene regulation and are wildly enriched in exosomes. They regulate the early stages of pregnancy.[17] Exosomal miRNAs derived from the endometrium promote embryo implantation by regulating the functions of trophoblasts.[18] For example, in the estrous cycle of cows, miRNA (miR)-1290 was expressed at a higher level on day 7 than day 3. But so far, there is no report on the role of miR-1290 in embryo implantation.[19] Successful pregnancy relies on appropriate regulation of the uterus and the development of the embryo. Synchronous communication is vital for successful embryo implantation. As such, this paper hypothesized that placental trophoblast cell-derived exosomal miRNAs regulate the interaction between embryo and endometrium by influencing endometrial states. To cross examine this idea, we subsequently confirmed that trophoblast-derived exosomal miRNA promotes the EMT process in the endometrial epithelial cells. Besides, exosomal miRNAs regulate the uterine environment, such as inflammatory conditions and angiogenesis. Our results provided a new mechanism and understanding that embryonic trophoblast-derived exosomes regulate embryo implantation.

RESULTS

Characterization of exosomes isolated from human extravillous trophoblast cells and their incorporation into endometrial cells

The exosomes from human extravillous trophoblast HTR8/SVneo cells were isolated and identified. The micrographs showed that the vesicles were round or saucer shaped as previously described (Figure 1A). Nanoparticle tracking analysis (NTA) revealed that the size of most EVs was less than 200 nm with a mean size of 131.7 nm (Figure 1B); this was consistent with the proposed size for exosomes. The western blotting analysis confirmed the expression of CD63, CD9, Alix,
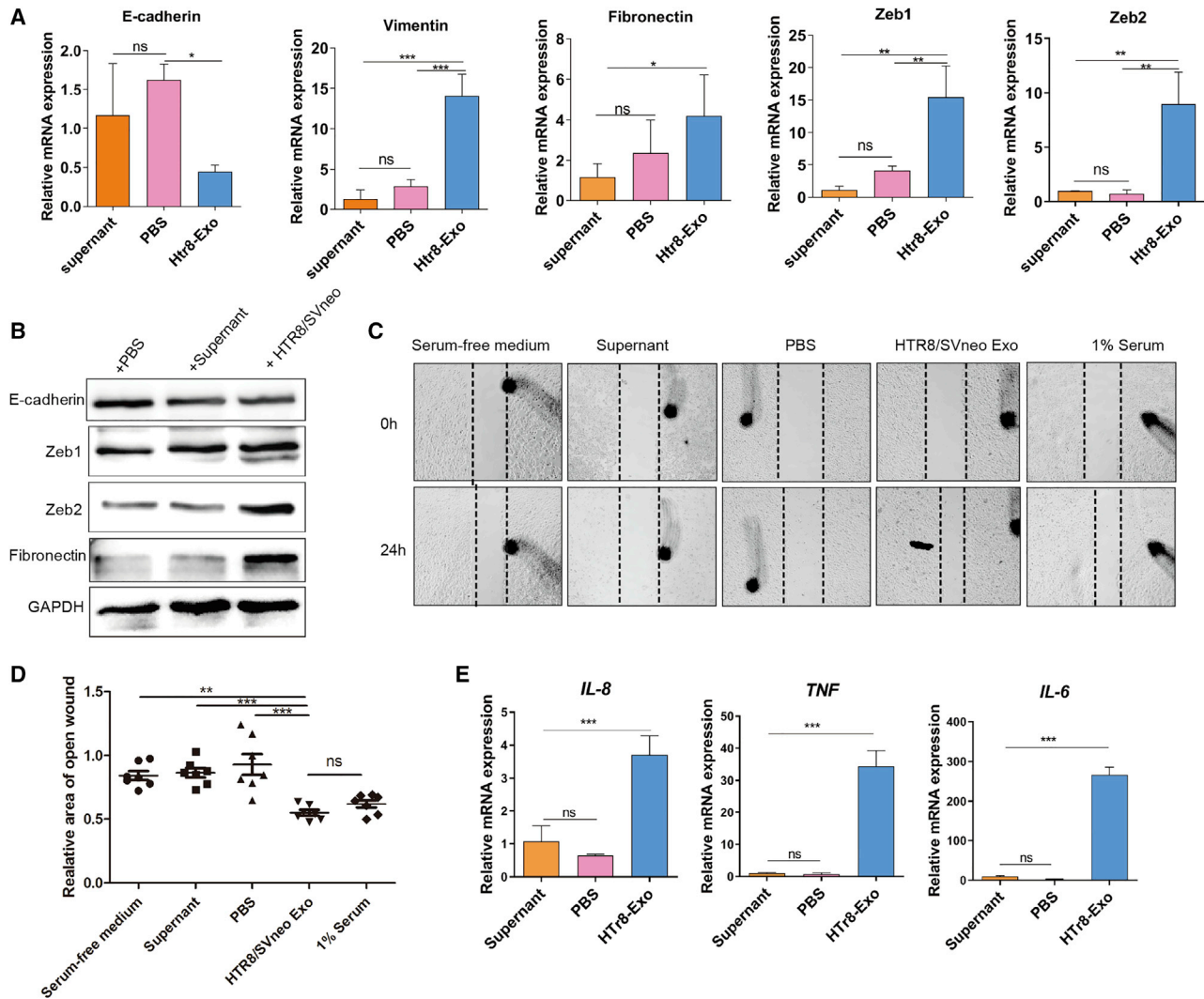
primarily focused on the effect of endometrium-derived exosomes; therefore, whether the exosomes released by trophoblasts promote the embryo implantation process remains unclear.

Figure 2. Trophoblast cell-derived exosomes promote the epithelial-mesenchymal transition (EMT) of endometrial cells

(A) The mRNA expression level of EMT markers (E-cadherin, vimentin, fibronectin, Zeb1, and Zeb2) was detected by qRT-PCR in endometrial cells after incubating with trophoblast cell-derived exosomes. (B) Protein level of EMT markers in endometrial cells after treatment with exosomes. (C and D) The results of the wound-healing assay showing the effects of exosomes on migration of endometrial cells; n = 7. Serum-free medium, supernatant, and PBS were used as NC; 1% serum as a positive control. (E) TNF, IL-6, and IL-8 mRNA levels were detected by qRT-PCR in endometrial cells after incubating with exosomes. *p < 0.05; **p < 0.01.

TSG101, and HSP70 (exosome protein markers) but not CANX in the exosome fraction (Figure 1C). These results confirm that the isolated nanoparticles were purified exosomes. Next, Dil-labeled exosomes were incubated with HEC-1-A cells to assess the capacity of endometrial cells to take up exosomes *in vitro*. The fluorescence results showed that Dil-stained exosomes were incorporated into HEC-1-A cells, and the fluorescent signal increased with incubation time (Figure 1D).

Extravillous trophoblast cell-derived exosomes induced endometrial cell EMT and expression of proinflammatory cytokines

The EMT process is vital for the endometrial function and successful decidualization, regeneration, and embryo implantation. Here, EMT

markers, E-cadherin, vimentin, fibronectin, Zeb1, and Zeb2, were detected using qRT-PCR and western blot to confirm whether trophoblast cell-derived exosomes promote the EMT process. The results revealed that the expression of E-cadherin (epithelial marker) in HEC-1-A cells was significantly lower after treatment with exosomes than PBS or the supernatant treatment group (Figure 2A). However, the expression of mesenchymal markers (vimentin, fibronectin, Zeb1, and Zeb2) was significantly upregulated in endometrial cells with exosome treatment (Figure 2A). The protein level results were consistent with mRNA levels (Figure 2B). Moreover, wound-healing assays revealed that the migration of endometrial cells was maintained in the serum-free medium. Cells treated with PBS or supernatant exhibited minimal migration 24 h after wounding, whereas those
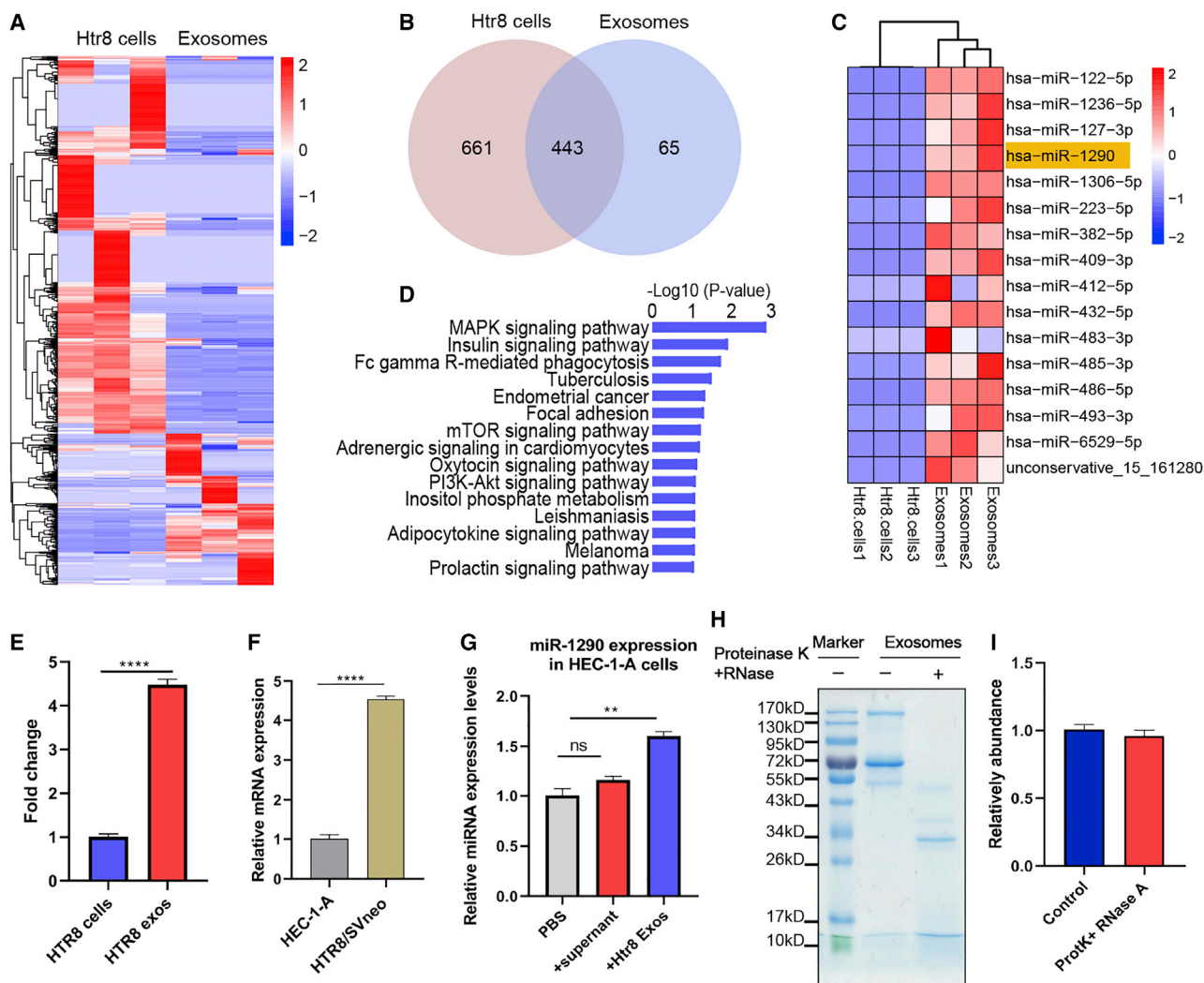
Figure 3. miR-1290 is highly enriched in trophoblast cell-derived exosomes

(A) Heatmap showed the differentially expressed miRNAs between HTR8/SVneo cells and exosomes. (B) Overlapping results of miRNAs in indicated groups. (C) The most significantly enriched miRNAs in trophoblast cell-derived exosomes. (D) Enriched pathways (top 15) of differentially expressed miRNA-predicted target genes in exosomes. (E) qPCR verification of miR-1290 in HTR8/SVneo cells and exosomes. (F) The expression of miR-1290 in endometrial cells (HEC-1-A) and trophoblast cells (HTR8/SVneo). (G) The detection of miR-1290 expression level in HEC-1-A cells after treatment with PBS, supernatants, and exosomes. (H) Coomassie blue staining showed the total protein level in exosome-enriched fractions when treated by proteinase K and RNase A. (I) miR-1290 levels detected from exosome-enriched fraction samples with and without the pretreatment of proteinase K and RNase A. *$p < 0.05$; **$p < 0.01$.

treated with trophoblast-derived exosomes displayed a comparable migration capacity to cells that received 1% serum stimulation (Figures 2C and 2D). These results suggest that placental trophoblast cell-derived exosomes could promote EMT in endometrial cells. The qRT-PCR results showed that the expression of tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-8 was significantly increased in exosomes treating HEC-1-A cells compared with the control group (Figure 2E). Altogether, these findings indicate that exosomes derived from extravillous trophoblast cells facilitate the EMT process in the endometrium and stimulate uterine inflammation for successful embryo implantation during early pregnancy.

Placental trophoblast cell-derived exosomes selectively load hsa-miR-1290

miRNAs play critical regulatory roles during early pregnancy and are abundant in exosomes. We investigated the miRNA profiles in trophoblast cells, and exosomes were detected by miRNA sequencing (miRNA-seq). Differentially expressed miRNAs between trophoblast cells and exosomes based on |log FC| > 2 (p values < 0.05) are shown in Figure 3A. Among the differentially expressed miRNAs, 443 miRNAs were co-expressed in cells and exosomes. A total of 661 and 65 miRNAs were specifically presented in trophoblast cells and exosomes, respectively (Figure 3B).

Therefore, 16 miRNAs with the most significant fold change (|logFC| > 8, p values < 0.01) in exosomes were focused (Figure 3C). A total of 148 target genes were selected for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. The KEGG pathway analysis showed that the target genes of those exosomal miRNAs were primarily enriched in the mitogen-activated protein kinase (MAPK) signaling pathway, focal adhesion, mTOR signaling pathway, phosphatidylinositol 3-kinase (PI3K)-AKT signaling pathway, etc. (Figure 3D). Reports indicate that miR-1290 regulates EMT during multiple biological processes.[20] Subsequently, miR-1290 was verified by qRT-PCR (Figure 3E), and the result was consistent with the sequencing data. The expression of miR-1290 was detected in trophoblast and endometrial cells. The findings indicate that HTR8/SVneo cells possess a significantly higher level of miR-1290 compared to HEC-1-A cells (Figure 3F). These data suggest that trophoblast cell-derived exosomes enriched miR-1290. Furthermore, to investigate the specific and selective load of miR-1290 in exosomes, HEC-1-A cells were incubated with exosomes than the level of miR-1290 in HEC-1-A was detected. miR-1290 was highly expressed in HEC-1-A cells treated with exosomes (Figure 3G). The degradation assay was performed using proteinase K and RNase A before RNA extraction to confirm the origin of miR-1290. As shown in Figure 3H, Coomassie blue staining results confirmed that the proteins in exosome-enriched fractions were largely degraded by proteinase K. RNase A treatment was used to degrade free and attached miRNAs in exosome fractions. The qRT-PCR results showed that the miR-1290, enriched in the exosome fraction, could not be degraded by the pretreatment of proteinase K and RNase A (Figure 3I). This suggests that miR-1290 was effectively protected by the exosomal membrane. These data, along with the previous results, suggest that miR-1290 is specifically loaded in trophoblast-derived exosomes and transported by endometrial cells.

Extravillous trophoblast cell-derived exosomal miR-1290 facilitates endometrial cell EMT

The above findings indicate that trophoblast-derived exosomes promote the migration and EMT process in HEC-1-A cells, whereas miR-1290 was enriched in these exosomes. However, the exact role of miR-1290 in EMT of endometrial cells remained unclear. Therefore, miR-1290 was exogenously overexpressed by transfecting mimics in HEC-1-A cells. mRNA levels of EMT markers were first detected, and the experiment *in vitro* revealed that E-cadherin was downregulated in HEC-1-A cells with mimic treatment. In contrast, vimentin, fibronectin, Zeb1, and Zeb2 were upregulated (Figure 4A). An inhibition experiment was also performed in HEC-1-A cells. As a consequence, E-cadherin was upregulated when miR-1290 was suppressed by an inhibitor in HEC-1-A cells. Nevertheless, no difference was noted in mesenchymal markers of the inhibitor group versus negative control (NC) (Figure 4A). Overexpression of miR-1290 upregulated the expression of mesenchymal biomarkers and simultaneously downregulated the protein levels of E-cadherin (Figure 4B). Moreover, trophoblast cell-derived exosomes overexpressing miR-1290 significantly promote the EMT of HEC-1-A cells compared

with the control or inhibited group (Figure S1). A wound-healing assay showed that overexpressed miR-1290 increased the migration capacity of HEC-1-A cells with a small opening wound area (Figures 4C and 4D). Similar results were observed when migration was measured in Transwell (migration) assays (Figure S2). Overall, these findings suggest that miR-1290 promotes EMT in HEC-1-A cells. Further, the expression of IL-6 and IL-8 was upregulated in HEC-1-A cells transfected with miR-1290 mimics. No difference was noted in TNF-α expression between the NC group and the transfected group (Figure 4E).

Extravillous trophoblast cell-derived exosomal miR-1290 promotes angiogenesis *in vitro*

Placental blood vessels are an important link between the maternal uterus and fetus in mammalian pregnancies. Specifically, it is accompanied by a rich angiogenesis process during early implantation. Previous studies indicate that exosomes derived from the maternal endometrium promote angiogenesis. This study explored whether trophoblast cell-derived exosomes promote angiogenesis. Besides, a tube-formation experiment was performed in human umbilical vein endothelial cells (HUVECs) treated with trophoblast-derived exosomes or PBS/supernatant. Consequently, exosomes promote tube formation *in vitro* (Figure 5A). Detection of migration and proliferation ability of HUVEC revealed that the treatment of trophoblast cell-derived exosomes increased migration and proliferation of HUVEC compared to those treated with PBS or supernatant (Figures 5B and 5C). These findings confirm the contribution of trophoblast-derived exosomes to angiogenesis. We further examined whether exosomal miR-1290 is implicated in angiogenesis. HUVEC was transfected with the miR-1290 mimic and its inhibitors, and consequently, the *in vitro* effect on angiogenesis was observed. The results showed that, unlike the control group, overexpression of miR-1290 promoted tube formation (Figure 6A) and stimulated the migration and proliferation of HUVECs (Figures 6B and 6C). Nonetheless, no significant difference was noted between the group transfected with the miR-1290 inhibitor and the control group. This potentially indicates that miR-1290 is involved in this process but does not play a significant role since it is a multifactorial process in angiogenesis.

Exosomal miR-1290 regulates the EMT of endometrial cells by targeting *LHX6*

A target gene of miR-1290 in endometrial cells was analyzed to further investigate the mechanism of exosomal miR-1290 in regulating the EMT process. miR-1290 regulates the expression of the LHX6 gene by targeting its 3′ UTR.[20] To identify the targets of exosomal miR-1290 in HEC-1-A cells, the binding site of miR-1290 and LHX6 was searched via TargetScan (Figure 7A). As shown in Figure 7B, miR-1290 mimics caused a significant decrease in the luciferase activity, and mutation of the miR-1290 binding site inhibited the knockdown effect (Figure 7B). As shown in Figure 6C, miR-1290 was successfully overexpressed or inhibited in HEC-1-A cells by transfecting the miR-1290 mimic or inhibitor (Figure 7C). Then, LHX6 expression could be downregulated in HEC-1-A cells by
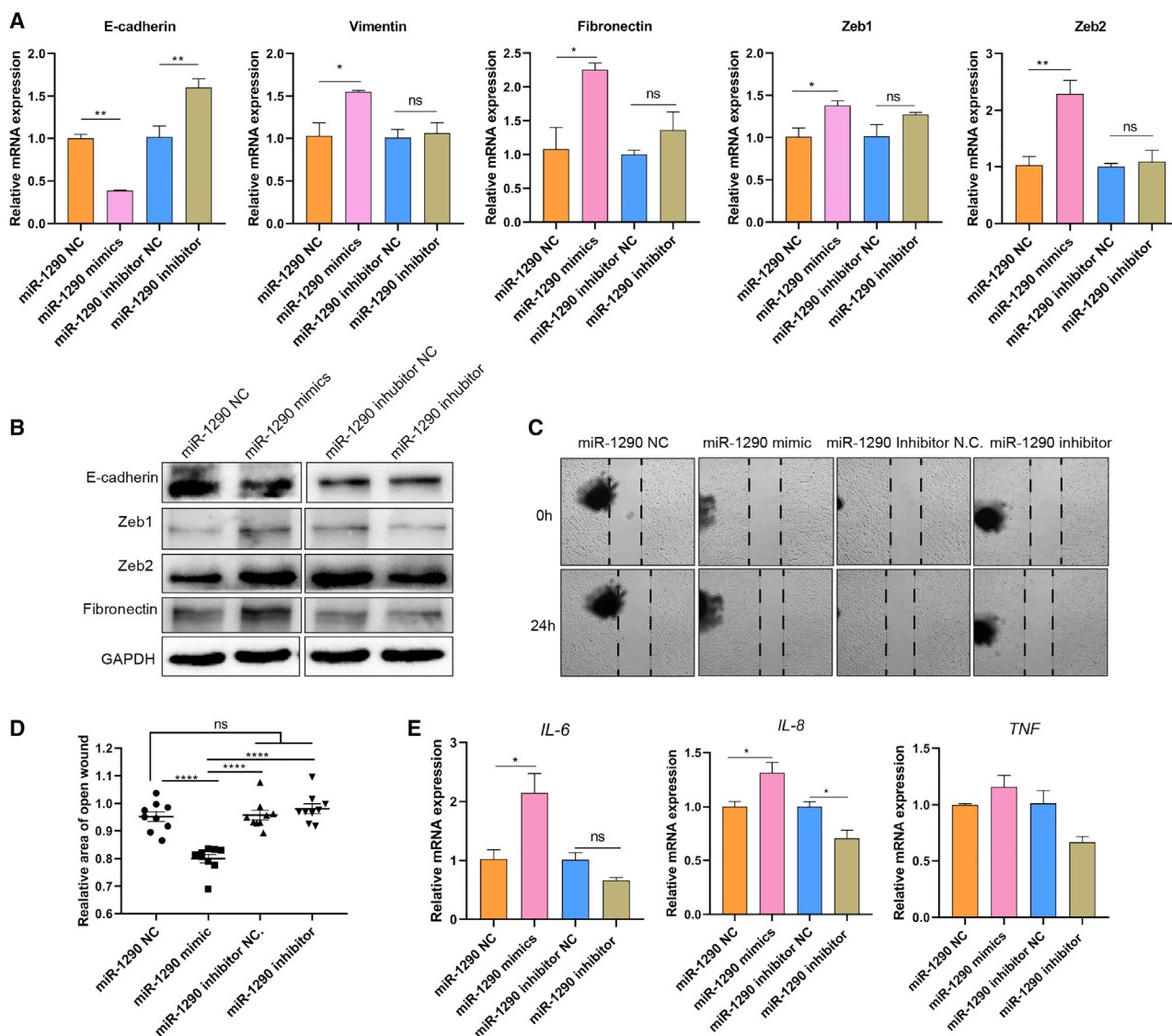
Figure 4. miR-1290 regulates the EMT process of HEC-1-A cells

(A) The expression of EMT markers (E-cadherin, vimentin, fibronectin, Zeb1, and Zeb2) in HEC-1-A cells after transfecting miR-1290 mimics was examined by qRT-PCR. (B) Immunoblotting assay of EMT protein markers in endometrial cells after transfecting miR-1290 mimic or normal control. (C and D) Migration assay of HEC-1-A cells transfected miR-1290 mimic or normal control. Relative open areas were counted, and representative images were shown. (E) mRNA levels of TNF, IL-6, and IL-8 were detected by qRT-PCR in endometrial cells after incubating with miR-1290 mimics or NC. *p < 0.05; **p < 0.01.

miR-1290 or exosomes derived from trophoblast cells at mRNA and protein levels (Figures 7D–7F). These findings reveal that LHX6 is a direct target of miR-1290 in HEC-1-A cells. Subsequently, to determine the function of LHX6 in the EMT process of endometrial cells, the LHX6 expression with small interfering RNAs (siRNAs) was knocked down in HEC-1-A, and the effect was identified by qRT-PCR (Figure 8A). The results suggest that 100 pmol concentration of LHX6-siRNA exhibited the best effect, and subsequent experiments were performed at this transfection concentration. The expression of E-cadherin and vimentin in HEC-1-A was detected in mRNA

and protein levels. E-cadherin was downregulated, and vimentin was upregulated in HEC-1-A cells when LHX6 was knocked down (Figures 8B and 8C). Also, LHX6 was downregulated when the LHX6-siRNA treated with HEC-1-A was used (Figure 8D). A wound-healing assay was performed to detect the migration ability of HEC-1-A cells. As shown in Figure 8E, HEC-1-A cells treated with LHX6-siRNAs exhibited enhanced motility. Similar results were reported when migration was measured in Transwell (migration) assays (Figure S3). Overall, these data suggest that LHX6 is a direct downstream target of miR-1290 and mediates the EMT process of HEC-1-A cells.
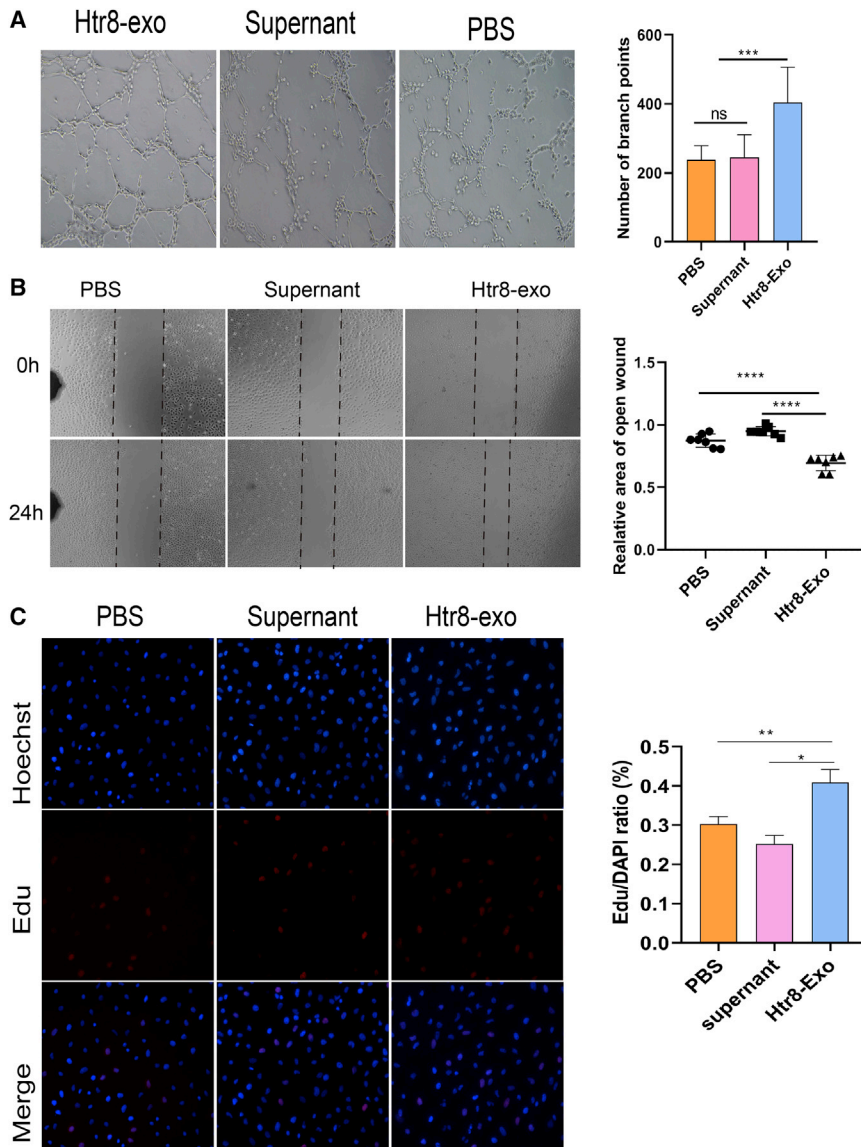
Figure 5. Trophoblast cell exosomal miR-1290 promotes angiogenesis *in vitro*

(A) Screening for enhanced HUVEC tube formation *in vitro* after treatment with trophoblast cell-derived exosomes, PBS, or supernatants, and evaluation of number of branch points was quantitated. (B) The migration ability of HUVEC was measured by a wound-healing assay after treating with exosomes or PBS. (C) The effect of trophoblast cell-derived exosomes on the proliferation ability of HUVEC.

porting embryo implantation.[18] Nonetheless, limited evidence is available on the effects of embryo-derived exosomes on the endometrium. Although significant studies suggest that the maternal uterus promotes successful pregnancy, the role of embryonic factors in the implantation process is vital. Embryo-derived exosomes in the different stages of development could be absorbed by human primary endometrial epithelial cells and vesicles with high histocompatibility leukocyte antigen (HLA)-G from day 3 to day 5 of development in *in vitro* culturing medium.[15] Desrochers et al.[22] discovered that embryonic stem cells communicate with trophoblasts through microvesicles and promote their implantation capacity. These findings suggest that exosomes derived from the embryo promote successful pregnancy. We also demonstrated that trophoblast-derived exosomal miRNAs regulate the transformation of endometrial cells to promote implantation.

Crosstalk between maternal uterus and embryo determines a successful pregnancy, specifically during the early stages. Accumulating evidence in mammals reveals that the implantation stage is vital for a successful pregnancy.[23] Most embryonic abortions occur during this period.[24] Despite artificial-assisted reproductive technology promoting the treatment of infertility and improving the pregnancy rate, its efficiency is significantly low. This is because the establishment of pregnancy depends on the bidirectional communications between mother and embryo. Therefore, it is necessary to investigate the mechanism of mother-fetal communication in early pregnancy. In the process of early embryo formation, the external trophoblast cells are essential for a connection between the embryo and the maternal uterus. They adhere to the uterine wall, migrate, and invade into the uterus, eventually forming the placenta.[9] This process is accompanied by blood vessel formation.[25,26] The interaction between the endometrium and trophoblasts is critical for a successful pregnancy. This paper demonstrated that placental

DISCUSSION

Recent studies have reported the role of exosomes in pregnancy.[8] Exosomal cargo, including miRNAs, are transported to the recipient cells, thereby affecting the physical state and function of these cells.[18,21] Notably, exosome-mediated maternal-fetal communication is bidirectional during pregnancy. EVs derived from the maternal uterus, i.e., exosomes, could be taken up by embryos.[16] Conversely, the exosomes secreted by the embryo could also be transported to the endometrium.[15] In our previous work, we demonstrated that receptive endometrial cell-derived exosomal miR-100-5p promotes the functions of trophoblasts (migration, invasion, and proliferation) during the implantation window. Other reports indicate that exosomal hsa-miR-30d isolated from uterine fluid could deliver to trophoblast cells, hence facilitating and sup-
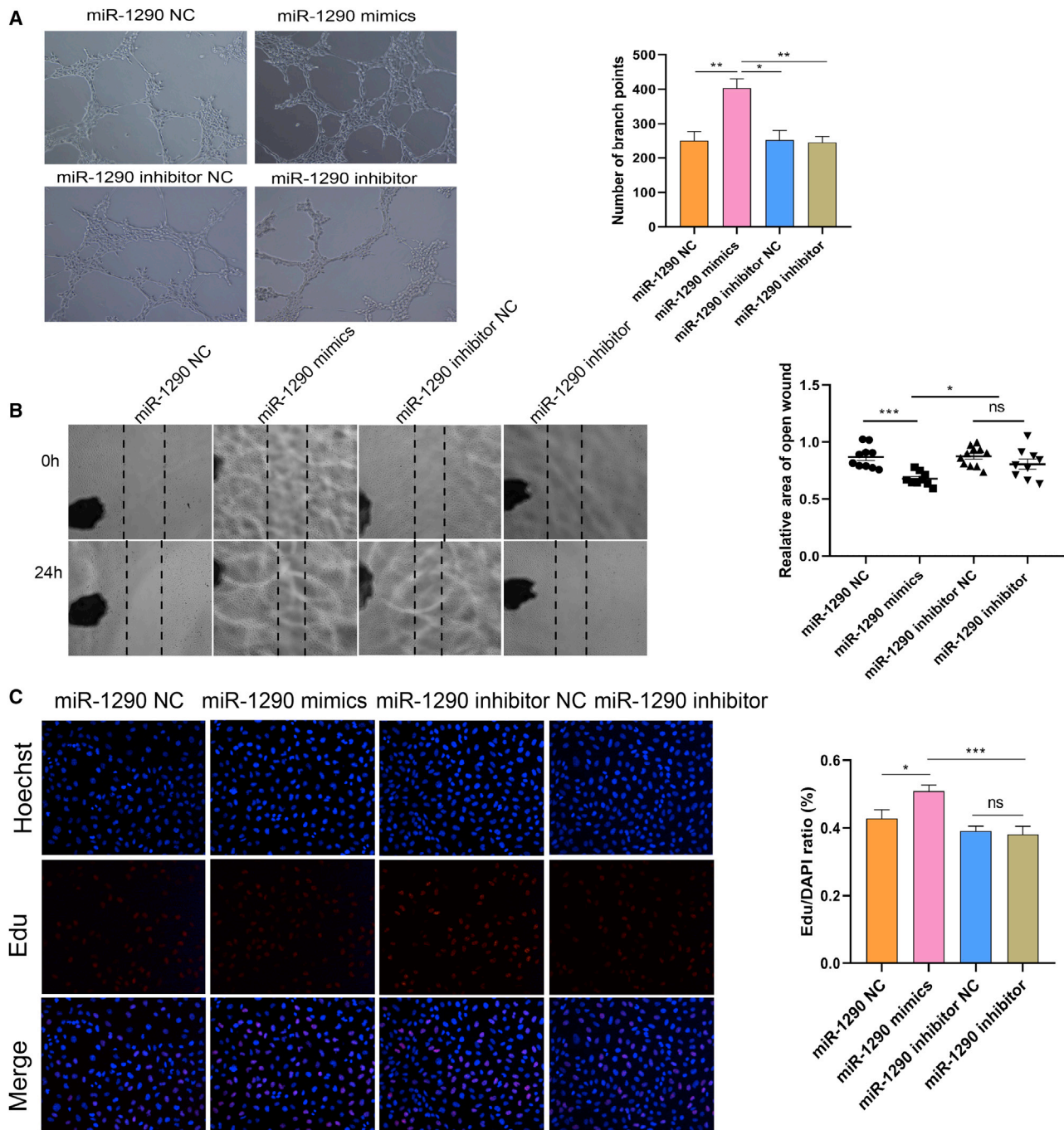
Figure 6. Trophoblast cell exosomal miR-1290 promotes angiogenesis *in vitro*

(A) Tube-formation analysis of HUVEC *in vitro* after transfection with miR-1290 mimics or inhibitors; the number of branch points was quantitated. (B) Wound-healing test to determine the migration ability of HUVECs after transfection with miR-1290 mimics or inhibitors, and the relative area of open wound was examined. (C) After transfecting with miR-1290 mimics or inhibitors, the proliferation ability of HUVEC was measured by Edu assay, and the ratio of Edu/DAPI was quantified and represented cell proliferation ability.

trophoblast cells secrete exosomes, which selectively load miRNAs, including miR-1290. Exosomal miR-1290 derived from trophoblasts promotes the EMT process and migration ability of endometrial epithelial cells by targeting LHX6. Additionally, we noted that trophoblast exosomal miR-1290 is implicated in angiogenesis and regulation of inflammation.
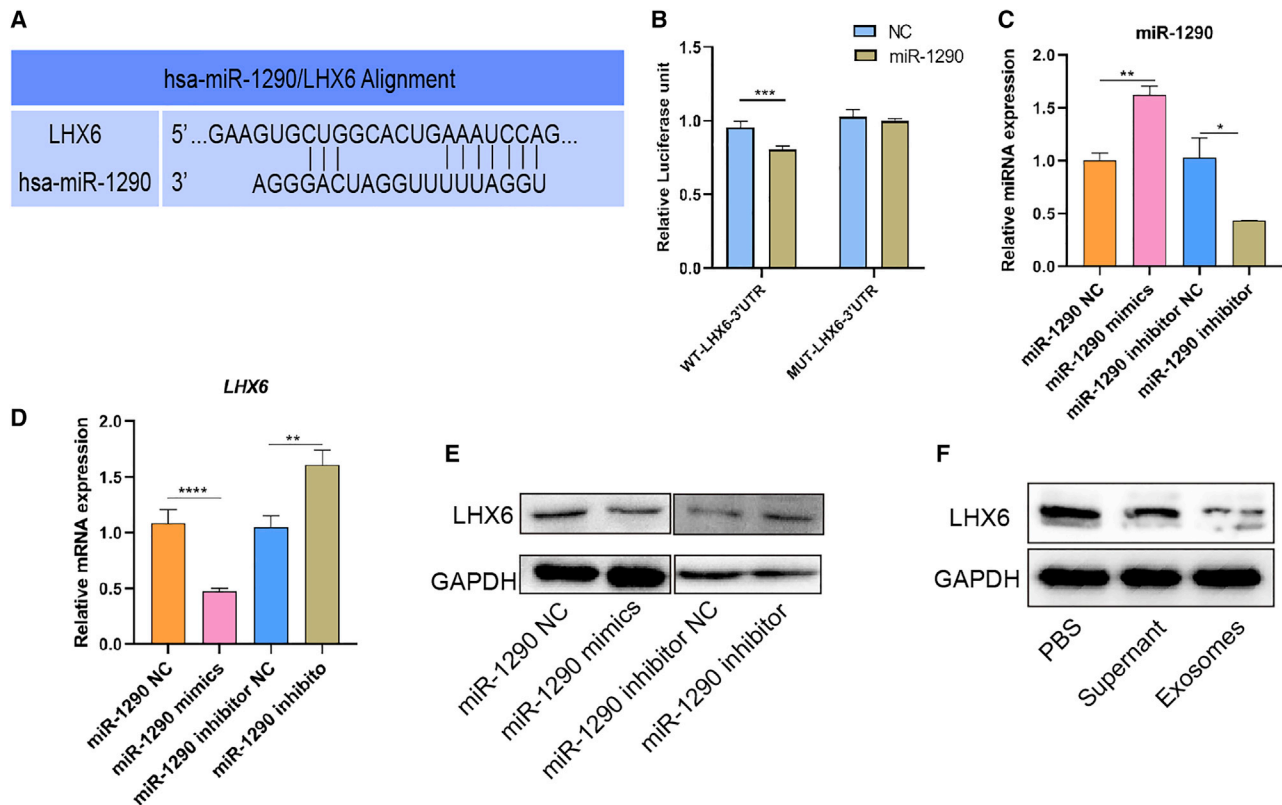
Figure 7. LHX6 is a direct downstream target of miR-1290 in the EMT process of endometrial cells

(A) The binding site of miR-1290 in the 3′ UTR area of the LHX6 gene. The site was searched via the TargetScan database. (B) Relative luciferase activity in HEC-1-A cells co-transfected with miR-1290 mimics, NC, and the wild-type (WT) or mutant (MUT) LHX6 3′ UTR vectors. (C) The expression of miR-1290 in HEC-1-A cells after transfecting with miR-1290 mimic or inhibitor. (D and E) qRT-PCR and immunoblotting assays of LHX6 expression in HEC-1-A cells treated with miR-1290 mimic or normal control. (F) Western blot analysis of LHX6 expression in HEC-1-A cells incubated with exosomes, PBS, or supernatants.

Endometrium undergoes regeneration and remodeling during the early stages of pregnancy; this is critical for successful reproduction. In early pregnancy, specifically during the implantation window, the endometrial epithelial cells transform from an epithelial to mesenchymal phenotype.[10] The EMT process in the endometrium facilitates the attachment and adhesion of blastocyst.[27,28] Notably, miRNAs influence uterine receptivity by regulating EMT.[17,29] For instance, miR-429 suppresses the EMT process by targeting protocadherin 8 (Pcdh8), a gene involved in cell-cell adhesion and cell morphology.[30] Besides physiological regulation of the uterus, embryo-derived factors regulate endometrial EMT, including the trophoblast-secreted platelet-derived growth factor AA and chemokine ligand 12.[31] Although EMT processes are crucial for successful embryo implantation, their exact regulatory role remains unclear. Here, we identified a set of miRNAs specifically loaded in trophoblast cell-derived exosomes. Among these miRNAs, exosomal miR-1290 regulates the EMT process in endometrial cells. Overexpressing miR-1290 upregulates mesenchymal markers in endometrial epithelial cells and enhances cell mobility. Arguably, this is the first study investigating the role of exosome-enriched miR-1290 in early pregnancy. miR-1290 is involved in multiple physiological and pathological signs of

progress, including cancer progression. It is highly expressed in several cancer cells and primarily promotes migration, invasion, and proliferation of cells.[20,32] In line with our findings, accumulating studies reveal that miR-1290 has a positive effect on EMT.[33] Our study uniquely revealed a novel origin of miR-1290, i.e., encapsulated in the trophoblast cell-derived exosome, and its contribution to successful implantation.

Previous studies confirmed that miR-1290 suppresses LHX6 expression by targeting its 3′ UTR. Bi et al.[34] reported that LHX6 inhibits the proliferation, invasion, and migration of breast cancer cells by suppressing the activation of PI3K/Akt/mTOR signaling. Besides, LHX6 could be regulated by miR-1237f in normoxic cells and to enhances the malignant phenotype by promoting EMT.[35] Nevertheless, studies on the functions of LHX6 during embryo implantation in the early stages of pregnancy have not matured. miR-1290 directly targets and suppresses the expression of LHX6, thereby promoting the EMT process and migration of endometrial epithelial cells. Additionally, together with previous findings, we speculated that trophoblast-derived exosomal miRNAs, including but not limited to miR-1290, are implicated in the regulation of uterine inflammation during early
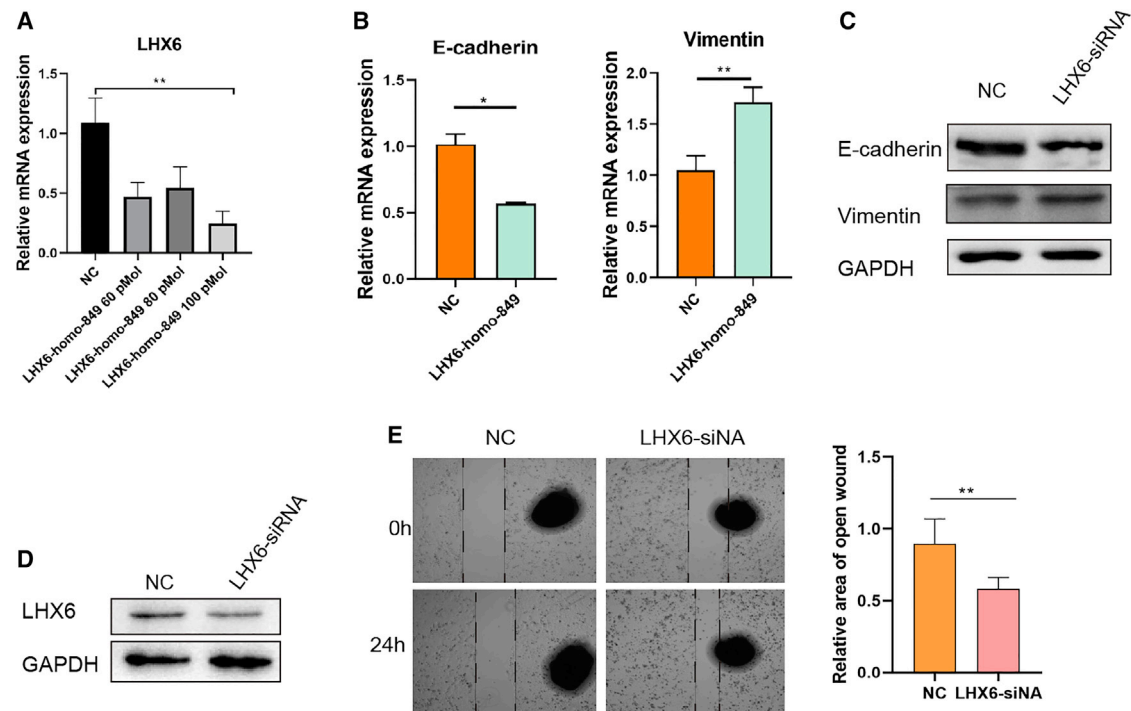
Figure 8. LHX6 is a direct downstream target of miR-1290 in the EMT process of endometrial cells

(A) HEC-1-A cells were transfected with different concentration of LHX-siRNA and normal control, and LHX6 expression was examined by qRT-PCR. (B and C) After knocking down LHX6 with siRNA, qRT-PCR and western blotting were performed to detect the expression of epithelial (E-cadherin) or mesenchymal marker (vimentin) in HEC-1-A cells. (D) After knocking down LHX6 with siRNA, western blotting was performed to detect the expression of LHX6 in HEC-1-A cells. (E) Migration assay of HEC1-1-A cells transfected with siRNAs targeting LHX6 or control.

pregnancy. This work also identified that exosomal miR-1290 promotes inflammation and angiogenesis. These results corroborate previous studies.[36–38] Trophoblast-derived exosomes are secreted to adjacent cells, promoting the regulation of the uterine immune environment and angiogenesis in early pregnancy, thereby establishing a close connection between fetus and uterus, hence a successful pregnancy.

In conclusion, these results indicate that trophoblast cell-derived exosomal miR-1290 promotes the EMT process of endometrial epithelial cells by targeting LHX6. Moreover, exosomal miR-1290 partly participates in the regulation of embryo implantation by promoting inflammation and angiogenesis, thereby enhancing the connection between the embryo and the maternal uterus. However, further investigation on the regulatory role of exosomal miR-1290 is essential; this will enhance the understanding of early pregnancy establishment. Furthermore, our findings provide an important reference value for infertility treatment. Meanwhile, inhibition of miR-1290 may serve as a potential contraceptive target.

MATERIALS AND METHODS

Cell culture

Human extravillous trophoblast cell line HTR8/SVneo was purchased from the American Type Culture Collection (ATCC), and human endometrium epithelial cell line HEC-1-A was purchased from the cell bank of the Chinese Academy of Science (Shanghai, China). HUVECs were a gift from Dr. Qin Zhiyuan, College of Pharmaceutical Sciences, Zhejiang University. HTR8/SVneo cells were cultured in RPMI-1640 medium supplemented with 5% fetal bovine serum (FBS). HEC-1-A was cultured in McCoy's 5A medium (Sigma), supplemented with 10% FBS. HUVECs were cultured in DMEM medium (Gibco), supplemented with 10% FBS (Gibco). Cells were maintained at 37°C, 5% CO_2.

Isolation of exosomes derived from HTR8/SVneo cells

In order to isolate exosomes from trophoblast cell culture medium, cells were cultured with 5% exosome-free FBS for 48 h. Exosome-depleted FBS was prepared by ultracentrifugation at 100,000 × g, 4°C overnight. The culture supernatant was collected and centrifuged at 500 × g for 10 min to remove dead cells, 2,000 × g for 20 min to remove cellular debris, and 10,000 × g for 35 min at 4°C to remove microvesicles. Next, the supernatant was filtered using a 0.22-μm filter and centrifuged at 120,000 × g for 75 min at 4°C. The pellets were re-suspended in PBS and centrifuged at 120,000 × g for 75 min at 4°C again. The final pellets containing exosomes were re-suspended with PBS for further usages. The supernatant in the final step of centrifugation was collected as a control. Protein concentration of exosomes was quantified using the

BCA Protein Assay (Beyotime; cat. no. P0010S). For the application of exosomes, 5×10^5 cells were incubated with 100 µg exosomes for the next research.

Exosome labeling
Exosomes were labeled with DiI dye (Beyotime; cat. no. C1036) to examine the uptake of exosomes by HEC-1-A cells in vitro. In brief, the labeled exosomes were incubated with HEC-1-A cells for 2 h, 4 h, 6 h, and 12 h, respectively. Then cells were fixed with 4% paraformaldehyde (Solarbio; cat. no. P1110), treated with 0.1% Triton X-100 (Solarbio; cat. no. T8200) for 30 min, and incubated with fluorescein isothiocyanate (FITC)-conjugated phalloidin (Solarbio; cat. no. CA1620). DAPI (Solarbio; cat. no. C0065) was used to mark nucleus. Images were obtained with a laser-scanning confocal microscope (Zeiss LSM 800; Zeiss, Germany).

Transmission electron microscopy (TEM)
To characterize exosomes, exosomes were placed onto the formvar carbon-coated copper grids at room temperature for 1 min. The excess suspension was removed using filter paper. Then exosomes were rinsed in double-distilled water thrice. Following, exosomes were stained using 2% uranylacetate at room temperature for 1 min. The grids were then dried in the air. Image was observed with a TecnaiG2 Spirit 120 kV transmission electron microscope operating at 120 kV (Thermo Fisher Scientific/FEI).

NTA
NTA was used to determine the size distribution and concentration of the exosomes. Exosomes were re-suspended 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 total proteins was measured by a BCA Protein Kit (Beyotime; cat. no. P0010S). SDS loading buffer was added to each sample, and the mixture was denatured for 10 min at 100°C. 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 37°C and incubated with the primary antibody including CD63 (Abcam; 1:1,000, cat. no. ab217345), CD9 (Diagbio; 1:1,000, cat. no. db919), Alix (Diagbio; 1:1,000, cat. no. db3856), HSP70 (Diagbio; 1:1,000, cat. no. db2396), Calnexin (ABclonal; 1:1,000, cat. no. A0803), GAPDH (Diagbio; cat. no. db106), E-cadherin (ABclonal; 1:1,000, cat. no. A11492), Zeb-1 (ABclonal; 1:1,000, cat. no. A5600), Zeb-2 (ABclonal; 1:1,000, cat. no. A5705), LHX6 (Proteintech; 1:1,000, cat. no. 21516-1-AP), and fibronectin (Diagbio; 1:1,000, cat. no. db2362) at 4°C overnight. Following, the membranes were incubated with secondary antibodies (ABclonal; 1:3,000) for 2 h. The blots were detected using BeyoECL Plus (Beyotime; cat. no. P0018S).

RNase and proteinase K treatment of trophoblast-derived exosomes
Proteinase K (Tiangen; cat. no. RT403) and RNase A (Tiangen; cat. no. RT405) were purchased from Tiangen Biotechnology in China. Trophoblast cell-derived exosomes were treated with proteinase K (final concentration, 100 µg/mL) for 30 min at 37°C and then treated with RNase A (final concentration, 10 µg/mL) for 15 min at 37°C.[39] Exosome-free RNA was used for RNA or protein extraction for the next research.

miRNA transfection to cells
miR-1290 mimics, mimic NCs, inhibitor NC, and inhibitor were purchased from GenePharma (Shanghai, China) and diluted with DEPC water. Before miRNA transfection, cells were seeded and cultured in a six-well plate for 24 h to achieve the proper growth density. Then, cells were transfected with miRNAs at a concentration of 50 nM using the HiPerFect Transfection Reagent (QIAGEN; cat. no. 301702) according to the manufacturer's procedures. After 48 h, cells were collected for the next research.

Edu assay
HUVECs were added into 12-well plates, and after 24 h of mimics or exosome treatment, cells were incubated with 10 µM Edu (Beyotime; cat. no. C0075S) for 2 h. Follow-up operations follow the manufacturer's instructions. Images were captured using a fluorescence microscope (Nikon).

RNA extraction and qRT-PCR
According to the manufacturer's protocols, total RNAs were extracted from the cells using Trizol reagent (Tiangen; cat. no. DP421). An antisense strand of RNAs was synthesized by the FastKing gDNA Dispelling RT SuperMix (Tiangen; cat. no. KR118). The relative expression of mRNA level was detected using the SuperReal PreMix Color (SYBR Green) qRT-PCR Kit (Tiangen; cat. no. FP215). miRNAs were isolated from cells and exosomes using the 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). The cDNA samples were run using the miRcute Plus miRNA qPCR Kit (SYBR Green; Tiangen; cat. no. FP411). GAPDH and U6 were used as internal controls. Relative miRNA and mRNA expression levels were assessed by the $2^{-\Delta\Delta Ct}$ method. All samples were tested in triplicate at least.

Exosomal miRNA-seq and bioinformatics analysis
miRNA components in HTR8/SVneo cells (n = 3) and exosomes (n = 3) were profiled by miRNA-seq analysis (Illumina HiSeq). A total amount of 1 ng−500 ng RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using the QIAseq miRNA Library Kit (QIAGEN, Frederick, MD, USA) following the manufacturer's recommendations, and index codes were added to attribute sequences to each sample. At last, library quality was assessed on the Agilent Bioanalyzer 2100 and qPCR. The clustering of the index-coded samples was performed on acBot Cluster Generation System using TruSeq PE Cluster

Kitv3-cBot-HS (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina HiSeq platform. miRNA-seq reads were normalized and quality assessed using fastp. The reads were mapped to reference genomes using miRdeep2 software. Then, the number of miRNA reads in each sample was counted. The DEseq2 package was used to identify DEMs with criteria as |log FC| > 2, and p ≤ 0.05. The miRTarBase database was used to predict the target genes of miRNA (https://mirbase.org/#:~:text=The%20miRBase%20database%20is%20a%20searchable%20database%20of,sequence%20of%20the%20mature%20miRNA%20sequence%20%28termed%20miR%29.), whereas the Gene Ontology (GO) and KEGG databases were performed to analyze gene functions.

Wound-closure (migration) assays
The wound-healing assay was used to determine the migration capacity of the cells. Cells were seeded in 6-well plates and cultured for 24 h until 70%–80% cell confluent monolayers and then wounded using a yellow pipette tip. After treatment with exosomes or miRNA mimics, the cells were allowed to migrate for 24 h. The area of wound was observed under the microscope, and relative mobility was analyzed using ImageJ software.

Transwell migration assay
The Transwell assay was used to establish the migration capacity of cells. HEC-1-A cells were pretreated with exosomes or miRNA mimics for 24 h before the cell motility assay. Thereafter, a Transwell with polycarbonate membranes (8 μm pore size; Corning) was employed. For migration assay, 1×10^5 HEC-1-A cells were re-suspended with McCoy's 5A medium without FBS and seeded into the inserts of the Transwell. Meanwhile, 500 μL McCoy's 5A medium containing 10% exosome-free FBS was added to the lower compartment. After incubation for 24 h, cell inserts were fixed and stained with 0.1% crystal violet solution (Solarbio; cat. no. G1064). Representative fields were photographed, and the cells were counted.

Tube-formation assay
In vitro angiogenesis experiments were determined by performing a tube-formation assay in Matrigel (Corning). A well of 48-well plates was pre-coated with 200 μL Matrigel and placed in a cell incubator at 37°C for 2 h. Then HUVECs with exosomes or miRNA treatment were added into 48-well plates and cultured for 8 h. Tube-formation fields were selected randomly and imaged with an optical microscope (Nikon). Tube length and total branch points were quantified using ImageJ software.

Dual-luciferase activity assay
A total of 6×10^4 HEC-1-A cells were seeded in 24-well plates, 24 h before transfection. Then, 500 ng pmirGLO vectors containing a wild-type or mutant fragment of the *LHX6* 3′ UTR (Promega, Madison, WI, USA), 50 nM miR-1290, or a scrambled control were co-transfected using Lipofectamine 2000 (Invitrogen). Luciferase activities of cellular extracts were measured 48 h after transfection using

a Dual-Luciferase Reporter Assay System (Promega). Transfection efficacy was normalized using Renilla luciferase activity.

Statistical analysis
In this study, the values were reported as mean ± SEM. Student's t test was used to compare the significant among two group. One-way analysis of variance (ANOVA) was performed to compare multiple (>2) means. p value ≤ 0.05 was considered to be significant as follows: **p ≤ 0.01, *p ≤ 0.05, and not significant (ns), p ≥ 0.05.

Data availability
All raw and processed next-generation sequencing data have been deposited with GEO under GEO: GSE182717. Processed data are available with this paper.

AUTHOR CONTRIBUTIONS
Q.T. and Z.W. designed the experiments. S.S. and Q.T. mainly conducted the experiments and analyzed the data. S.S., Q.T., and J.JL. wrote and revised the paper. D.C., S.W., J.YL., and K.C. were involved with the experiments. Z.W. organized and supervised the study.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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