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ORIGINAL ARTICLE

Basic Study

Effects of miR-214-5p and miR-21-5p in hypoxic endometrial epithelial-cell-derived exosomes on human umbilical cord mesenchymal stem cells

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Abstract

BACKGROUND

Thin endometrium seriously affects endometrial receptivity, resulting in a significant reduction in embryo implantation, and clinical pregnancy and live birth rates, and there is no gold standard for treatment. The main pathophysiological characteristics of thin endometrium are increased uterine arterial blood flow resistance, angiodysplasia, slow growth of the glandular epithelium, and low expression of vascular endothelial growth factor, resulting in endometrial epithelial cell (EEC) hypoxia and endometrial tissue aplasia. Human umbilical cord mesenchymal stem cells (HucMSCs) promote repair and regeneration of damaged endometrium by secreting microRNA (miRNA)-carrying exosomes. However, the initiation mechanism of HucMSCs to repair thin endometrium has not yet been clarified.

AIM

To determine the role of hypoxic-EEC-derived exosomes in function of HucMSCs and explore the potential mechanism.

METHODS

Exosomes were isolated from normal EECs (EEC-exs) and hypoxia-damaged EECs (EECD-exs), before characterization using Western blotting, nanoparticle-tracking analysis, and transmission electron microscopy. HucMSCs were cocultured with EEC-exs or EECD-exs and differentially expressed miRNAs were de-

termined using sequencing. MiR-21-5p or miR-214-5p inhibitors or miR-21-3p or miR-214-5p mimics were transfected into HucMSCs and treated with a signal transducer and activator of transcription 3 (STAT3) activator or STAT3 inhibitor. HucMSC migration was assessed by Transwell and wound healing assays. Differentiation of HucMSCs into EECs was assessed by detecting markers of stromal lineage (Vimentin and CD13) and epithelial cell lineage (CK19 and CD9) using Western blotting and immunofluorescence. The binding of the miRNAs to potential targets was validated by dual-luciferase reporter assay.

RESULTS

MiR-21-5p and miR-214-5p were lowly expressed in EECD-ex-pretreated HucMSCs. MiR-214-5p and miR-21-5p inhibitors facilitated the migratory and differentiative potentials of HucMSCs. MiR-21-5p and miR-214-5p targeted STAT3 and protein inhibitor of activated STAT3, respectively, and negatively regulated phospho-STAT3. MiR-21-5p- and miR-214-5p-inhibitor-induced promotive effects on HucMSC function were reversed by STAT3 inhibition. MiR-21-5p and miR-214-5p overexpression repressed HucMSC migration and differentiation, while STAT3 activation reversed these effects.

CONCLUSION

Low expression of miR-21-5p/miR-214-5p in hypoxic-EEC-derived exosomes promotes migration and differentiation of HucMSCs into EECs *via* STAT3 signaling. Exosomal miR-214-5p/miR-21-5p may function as valuable targets for thin endometrium.

Key Words: Endometrial epithelial cells; Exosomes; Human umbilical cord mesenchymal stem cells; MiR-214-5p/miR-21-5p; Signal transducer and activator of transcription 3

Core Tip: Thin endometrium is a primary cause of repeated implantation failure and infertility. In this study, with the help of microRNA sequencing, we investigated the role of hypoxic endometrial epithelial cell (EEC)-derived exosomes in cell function of human umbilical cord mesenchymal stem cells and explored the potential mechanism. Eventually, we found that lowly-expressed miR-21-5p and miR-214-5p in hypoxic EEC-derived exosomes promoted migration and differentiation of human umbilical cord mesenchymal stem cells into EECs *via* signal transducer and activator of transcription 3 signaling. Exosomal miR-214-5p and miR-21-5p may function as valuable targets for thin endometrium.

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INTRODUCTION

The endometrium is a dynamic, repetitively cycling tissue that mediates blastocyst implantation[1]. Thin endometrium is considered to be a major cause of unsuccessful embryo transfer, leading to long-term infertility[2]. Possible pathological causes of thin endometrium include radiation, infection, history of uterine surgery, and Asherman syndrome[3]. Numerous treatment strategies have been proposed for treating thin endometrium[4], although their efficacy is still limited. Further research is required to improve the therapeutic strategy for thin endometrium.

Exosomes are nanosized structures released by normal and pathological cells, which are responsible for delivering bioactive cargoes such as nucleic acids, lipids, and proteins[5]. Exosomes play a role in regulating cell function in disorders of the endometrium[6]. MicroRNAs (miRNAs) are noncoding small RNAs comprised of about 22 nucleotides, which bind to target mRNAs for transcription and translation regulation[7]. Exosomes-derived miRNAs are related to various cellular processes, including cell proliferation, differentiation, and migration[8]. In ovarian endometriosis, exosomes derived from endometrial epithelial cells (EECs) carry miRNA-30c and repress cell migration and invasion[9]. Exosomes from bovine EECs ensure the development of trophoblast cells *via* secreting miR-218[10]. Additionally, exosomes can be used to deliver miR-218 from EECs into the uterine microenvironment to modulate immune responses [11].

Mesenchymal stem cells (MSCs) are multipotent cells possessing the capabilities of self-renewal and multidirectional differentiation[12]. Recent clinical applications use umbilical cord-derived MSCs (ucMSCs) for treating human diseases, including metabolic/endocrine-related diseases and reproductive disorders[13]. Previous studies have validated the beneficial role of ucMSCs in disorders of the endometrium. UcMSC transplantation contributes to the repair of endometrial damage and restoration of fertility[14]. In rats, ucMSC transplantation facilitates the restoration of thin endometrium[15], and Pluronic F-127-encapsulated human ucMSCs (HucMSCs) promote the angiogenesis and regeneration of thin endometrium[16]. Exosomes derived from HucMSCs repair damaged EECs[17,18]. HucMSCs can migrate

to the injured endometrium and facilitate restoration of thin endometrium[15]. MSCs derived from human umbilical cord Wharton's jelly can differentiate into EEC-like cells[19]. Nonetheless, the role of EEC-released exosomes in the function of HucMSCs has not been elucidated. Here, we investigated whether exosomes derived from injured EECs could induce HucMSC migration and differentiation into EECs, thereby repairing the damaged endometrium. We explored the biological role of hypoxic-EEC-derived exosomes in the function of HucMSCs, as well as the potential molecular mechanism involved.

MATERIALS AND METHODS

Cell culture

HucMSCs at passage 3 were obtained from Cellverse Bioscience Technology Co. Ltd. (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F12) (03.2001C, EallBio, Beijing, China) with 10% fetal bovine serum (FBS) (SH30406.05, HyClone, Marlborough, MA, United States). Primary human EECs were bought from Cellverse Bioscience Technology and maintained in DMEM (11965092, Gibco, Carlsbad, CA, United States) containing 10% FBS. Streptomycin (100 U/mL) and 100 mg/mL penicillin were added to the medium. Cells were cultured in normoxic (21% O_2, 75% N_2, and 5% CO_2) conditions at 37 °C. HucMSCs at passages 4-6 were used for the next experiments, as previously described[18].

EEC hypoxia injury model

EECs were grown to about 70% confluence in normoxic air. To induce hypoxia, cells were maintained in a modular incubator chamber in a humidified hypoxic condition (5% CO_2, 94% N_2, and 1% O_2) at 37 °C for 4 h. In the control group, EECs were only incubated in normoxic conditions.

Isolation and identification of exosomes

Exosomes in culture medium of hypoxia damaged-EECs (EECD-exs) or normal EECs (EEC-exs) were extracted by ultracentrifugation. EECs were maintained for 48 h with exosome-free DMEM. To remove cell debris, the medium was centrifuged (2000 × *g*) at 4 °C for 20 min, prior to 10000 × *g* at 4 °C for 30 min. A 0.2-μm filter was used to filter the supernatants. The supernatants were ultracentrifuged at 100000 × *g* at 4 °C for 1 h. The precipitate was resuspended in phosphate-buffered saline, followed by ultracentrifugation at 100000 × *g* for 1 h. Isolated exosomes were gathered with exosome precipitation reagent.

Exosome morphology was identified by transmission electron microscopy (TEM) (HT7800; Hitachi, Tokyo, Japan). Nanoparticle-tracking analysis (NTA) was performed to analyze the concentration and size of exosomes with an analyzer (ZetaVIEW S/N 252, ParticleMetrix, Germany). Specific markers of exosomes [CD81/tetraspanin-28 (Tspan-28) and CD63] were determined by Western blotting with corresponding primary antibodies against CD81/Tspan-28 (ab109201, Abcam, Cambridge, United Kingdom) and CD63 (ab134045, Abcam).

miRNA sequencing

Total RNA was isolated from cells, and an Agilent Technologies 2100 Bioanalyzer (CA, United States) was used to examine the quality of total RNA. The small RNA library was prepared by using the TruSeq small RNA library prep kit (RS-200-0036, Illumina, San Diego, CA, United States). Following multiplexing in equimolar amounts, indexed small RNA libraries were denatured and loaded for cluster generation on GAIIx flow cell lanes with cBot station and Illumina cluster generation kits. Differentially expressed miRNAs showing raw reads ≥ 5 in samples and $P < 0.05$ were chosen.

Cell transfection and treatment

HucMSCs were pretreated with EEC-exs or EECD-exs (5 μg/mL) for 24 h, which formed the EEC-ex + HucMSC or EECD-ex + HucMSC group, respectively. For cell transfection, the negative controls, miR-214-5p/miR-21-5p inhibitors, and miR-214-5p/miR-21-5p mimics were synthesized by Sangon Biotech, Shanghai, China. HucMSCs (2×10^4/well) were grown to 80% confluence in six-well plates. HucMSCs were transfected with normal control (NC) mimic, NC inhibitor, miR-214-5p/miR-21-5p inhibitors, or miR-214-5p/miR-21-5p mimics (30 nM/well) using jetPRIME (101000046, Polyplus, Illkrich, France). For inhibition or activation of signal transducer and activator of transcription 3 (STAT3), the STAT3 inhibitor Stattic (HY-13818, MedChemExpress, Junction, NJ) or agonist colivelin (HY-P1061, MedChemExpress) was added to HucMSCs.

Wound healing and Transwell assays

For the wound healing assay, cells were implanted in a six-well plate. A pipette tip was used to scratch the cell monolayer on the bottom of the plate to draw a gap between cells. Images were obtained at the indicated times to observe cell migration. Image-Pro Plus was used to quantify the number of migrating cells. For the Transwell assay, 4×10^4 cells were added to the upper Transwell chamber for incubation in culture medium without FBS, and culture medium supplemented with 10% FBS was added to the lower chamber. After 24 h, cells migrating to the lower chamber were fixed in 2% methanol, before staining with 5% crystal violet. The positively stained cells were imaged under a microscope (2103012, AOSVI, Shenzhen, China).

Western blotting

RIPA buffer with protease inhibitors (P0013B, Beyotime, Shanghai, China) was applied to extract proteins from the cells. Protein samples were separated on sodium-dodecyl sulfate gel electrophoresis, before transfer to polyvinylidene difluoride membranes (IPVH00010, Millipore, Burlington, MA). After blocking for 1 h at room temperature with non-fat milk (5%), the membranes were incubated with anti-STAT3 (4904, Cell Signaling technology, Danvers, MA), E3 SUMO-protein ligase PIAS3/protein inhibitor of activated STAT3 (PIAS3) (4164, Cell Signaling Technology), anti-p-STAT3 (9138, Cell Signaling Technology), anti-CK19 (ab76539, Abcam), anti-CD9 (ab236630, Abcam), anti-vimentin (5741, Cell Signaling Technology), anti-CD13 (ab108310, Abcam), and anti-β-tubulin (CW0098M, CWBIO, Beijing, China) at 4 °C overnight. On the following day, after rinsing with TBST, the membranes were incubated with secondary antibodies conjugated with horseradish peroxidase (5220-0336, KPL, Gaithersburg, MD, United States). Enhanced chemiluminescence solution (MA0186-2, Meilunbio, Dalian, China) was applied for visualizing the protein bands.

Immunofluorescence staining

The differentiation of HucMSCs into endometrial stromal cells (ESCs) and EECs were identified by staining with antibodies against the markers of epithelial cell lineage (CD9 and CK19), and against the markers of stromal lineage (CD13 and vimentin). After fixation in 4% paraformaldehyde, HucMSCs were blocked in 1% bovine serum albumin. Cells were incubated with anti-vimentin (ab92547, Abcam), anti-CD13 (ab108310, Abcam), anti-CK19 (ab76539, Abcam), or anti-CD9 (ab236630, Abcam) overnight at 4 °C, followed by incubation with secondary antibodies. Following counterstaining with 4′,6-diamidino-2-phenylindole, cells were observed by confocal fluorescence microscopy (ECLIPSE Ti2, Nikon, Tokyo, Japan).

Reverse transcription-quantitative polymerase chain reaction

TRIzol reagent (R1100, Solarbio, Beijing, China) was applied to isolate total RNA from cells, prior to the preparation of cDNA with total RNA (50 ng) in a reaction mixture (20 μL) using iScript cDNA Synthesis Kit (KR118, Tiangen, Beijing, China). Gene expression was quantified by performing reverse transcription-quantitative polymerase chain reaction (RT-qPCR) with SYBR Green (FP205, Tiangen) and specific primers (Table 1), and optimized on the Real-Time PCR Detection System (ABI7500, Thermo Scientific, Waltham, MA, United States). RNA expression was determined using the $2^{-\Delta\Delta CT}$ method. For each sample, the expression of miRNA and mRNA was normalized to U6 and GAPDH, respectively.

Dual-luciferase reporter assay

TargetScan and miRDB were utilized to predict the target genes of miR-214-5p or miR-21-5p. The 3′-untranslated region (UTR) sequence of PIAS3 or STAT3 containing a binding site for miR-214-5p or miR-21-5p was inserted into psiCHECK-2 vector (General Bio, Anhui, China) to generate PIAS3-WT or STAT3-WT. The 3′-UTR sequence of PIAS3 or STAT3 was mutated for construction of PIAS3-Mutant or STAT3-Mutant. Cells were seeded in a 24-well plate and incubated for 24 h. MiR-214-5p/miR-21-5p mimic and PIAS3/STAT3-WT or PIAS3/STAT3-Mutant were transfected into the cells. A dual-luciferase reporter system (RG028, Beyotime) was used to evaluate luciferase activity 24 h after transfection.

Statistical analysis

Results are described as the mean ± SD. One-way ANOVA was carried out for multiple comparisons. Student's *t* test was used for the comparisons between two groups. Statistical analyses were undertaken employing GraphPad Prism 7.0. Differences were considered significant at $P < 0.05$.

RESULTS

Identification of EEC-exs and EECD-exs

EECD-exs and EEC-exs were extracted, then TEM, NTA, and Western blotting were performed to identify the extracted exosomes. The vesicle-like exosomes (EEC-exs) were spherical on TEM imaging (Figure 1A). The isolated exosomes (EEC-exs) expressed typical positive markers (CD63 and CD81/Tspan-28) (Figure 1B). NTA data indicated that the exosomes (EEC-exs) were about 150 nm in size (Figure 1C). These results demonstrated the successful isolation of exosomes from culture medium of EECs.

EECD-exs promote HucMSC migration and differentiation into EECs

To determine the function of EECD-exs in the migration of HucMSCs, HucMSCs were cocultured with EEC-exs or EECD-exs. Compared with the EEC-ex + HucMSC group, the migration of HucMSCs was promoted after coculture with EECD-exs, exhibited by the wound healing assay (Figure 2A and B). The data indicated that EECD-exs promoted the migration of HucMSCs. EECD-ex coculture increased protein expression of the markers of epithelial cell lineage (CD9 and CK19), and decreased expression of the markers of stromal lineage (vimentin and CD13) in HucMSCs (Figure 2C), suggesting that EECD-exs promoted differentiation of HucMSCs into EECs.

Differentially expressed miRNAs in EECD-ex-cocultured HucMSC

To determine differentially expressed miRNAs in exosomes cocultured with HucMSCs, miRNA sequencing analysis was performed. Heat maps showed the miRNA profiles that were differentially expressed in the EECD-ex + HucMSC group compared with the EEC-ex + HucMSC group (Figure 3A). RT-qPCR showed low expression of miR-214-5p and miR-21-5p

Table 1 Primer sequences used in reverse transcription-quantitative polymerase chain reaction

Gene		Primer sequence (5'-3')
U6	F	ACGATACAGAGAAGATTAGCATGG
	R	AAATATGGAACGCTTCACGAA
hsa-miR-1303	F	CTCAACTGGTGTCGTGGAGT
	R	TCGGCAGGTTTTAGAGACGGGGTCT
	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCTCTCGT
hsa-miR-4521	F	CTCAACTGGTGTCGTGGAGT
	R	TCGGCAGGGCTAAGGAAGTCCTGTGCT
	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCAAACTG
hsa-miR-7974	F	CTCAACTGGTGTCGTGGAGT
	R	TCGGCAGGAGGCTGTGATGCTCTCCT
	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCGGGCTC
hsa-miR-210-3p	F	CTCAACTGGTGTCGTGGAGT
	R	TCGGCAGGCTGTGCGTGTGACAGCG
	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCTCAGCC
hsa-miR-31-5p	F	CTCAACTGGTGTCGTGGAGT
	R	TCGGCAGGAGGCAAGATGCTGGCATAGCTGT
	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCACAGCT
hsa-miR-214-5p	F	CTCAACTGGTGTCGTGGAGT
	R	TCGGCAGGTGCCTGTCTACACTTGC
	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCGCACAG
hsa-miR-21-5p	F	CTCAACTGGTGTCGTGGAGT
	R	TCGGCAGGTAGCTTATCAGACTGAT
	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCGTCAAC
PIAS3	F	CTGGGCGAATTAAAGCACATGG
	R	AAAGCGTCGTCGGTAAAGCTC
STAT3	F	ACCAGCAGTATAGCCGCTTC
	R	GCCACAATCCGGGCAATCT
GAPDH	F	ACGGATTTGGTCGTATTGGG
	R	GGGATCTCGCTCCTGGAAG

STAT3: Signal transducer and activator of transcription 3.

in the EECD-ex + HucMSC group, which was consistent with the data from miRNA sequencing (Figure 3B and C). RT-qPCR showed that expression of miR-1303, miR-4521, miR-7974, miR-210-3p, and miR-31-5p was inconsistent with the data from miRNA sequencing (Supplementary Figure 1). We propose that EECD-exs might deliver miRNA into HucMSCs, which leads to promotion of HucMSC migration and differentiation *via* regulating certain signaling pathways.

miR-21-5p or miR-214-5p inhibitor promotes HucMSC migration and differentiation into EECs

To explore whether miR-21-5p or miR-214-5p is implicated in regulating HucMSC function, miR-21-5p or miR-214-5p inhibitor was transfected into HucMSCs. The successful inhibitor of miR-21-5p or miR-214-5p was validated by RT-qPCR (Figure 4A and B). Transwell assay showed that HucMSC migration was enhanced by miR-21-5p or miR-214-5p inhibitor (Figure 4C and D). Similar results were obtained by wound healing assay (Figure 4E and F). MiR-21-5p or miR-214-5p inhibitor led to elevated expression of CD9 and CK19 and decreased expression of vimentin and CD13 in HucMSCs (Figure 4G and H). Immunofluorescence staining also showed that CD9 and CK19 were increased while CD13 and vimentin were decreased in HucMSCs after miR-21-5p or miR-214-5p silencing (Figure 4I and J). Compared with the NC inhibitor control, after transfection with miR-21-5p or miR-214-5p inhibitor, the alizarin red staining of HucMSCs after induction of osteogenic differentiation was significantly weakened, and the alginate-like polymers level significantly

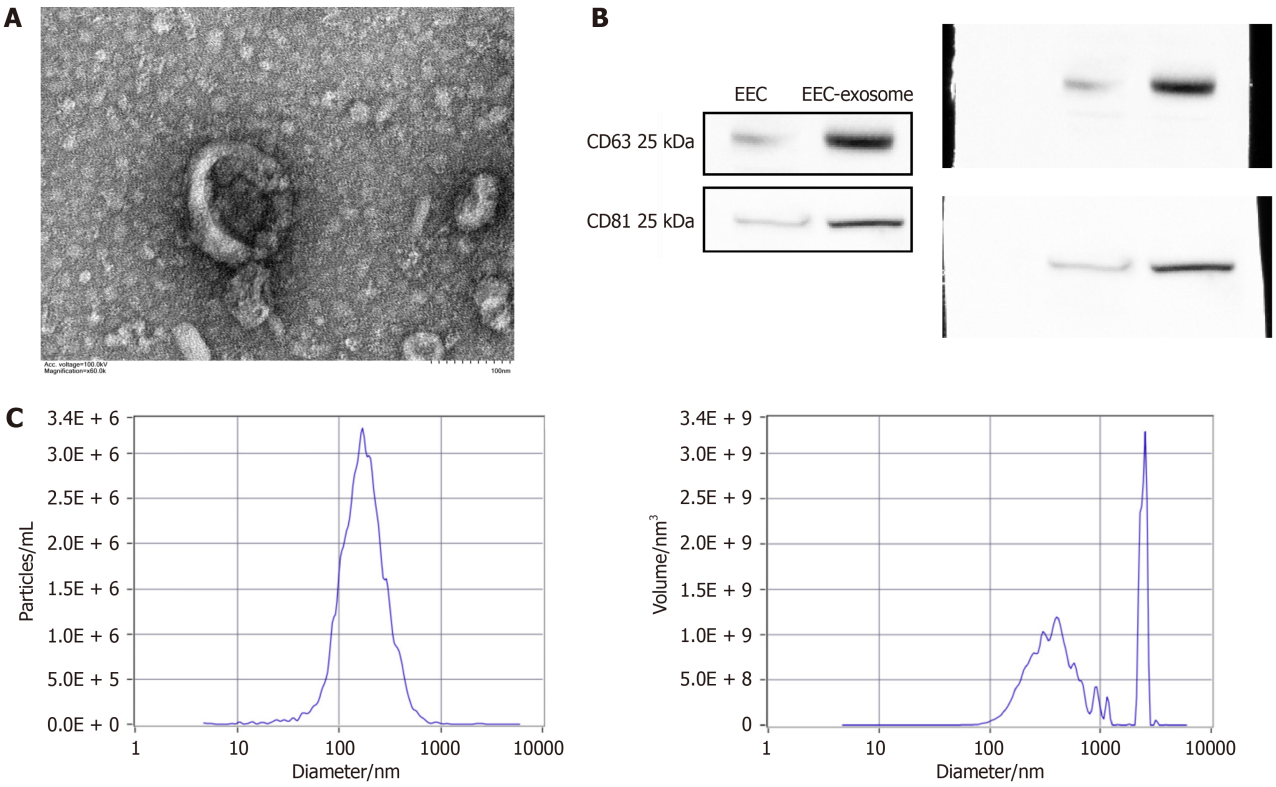


Figure 1 Identification of exosomes derived from endometrial epithelial cells and hypoxia-damaged endometrial epithelial cells. A: Endometrial epithelial cell exosomes were isolated from normal endometrial epithelial cells (EEC-exs) and exosomes derived from hypoxia-damaged endometrial epithelial cells were observed by transmission electron microscopy; B: Protein expression of CD63 and CD81/tetraspanin-28 in EEC-exs was measured by Western blotting; C: The particle number and average diameter of EEC-exs revealed by nanoparticle-tracking analysis. EEC: Endometrial epithelial cell.

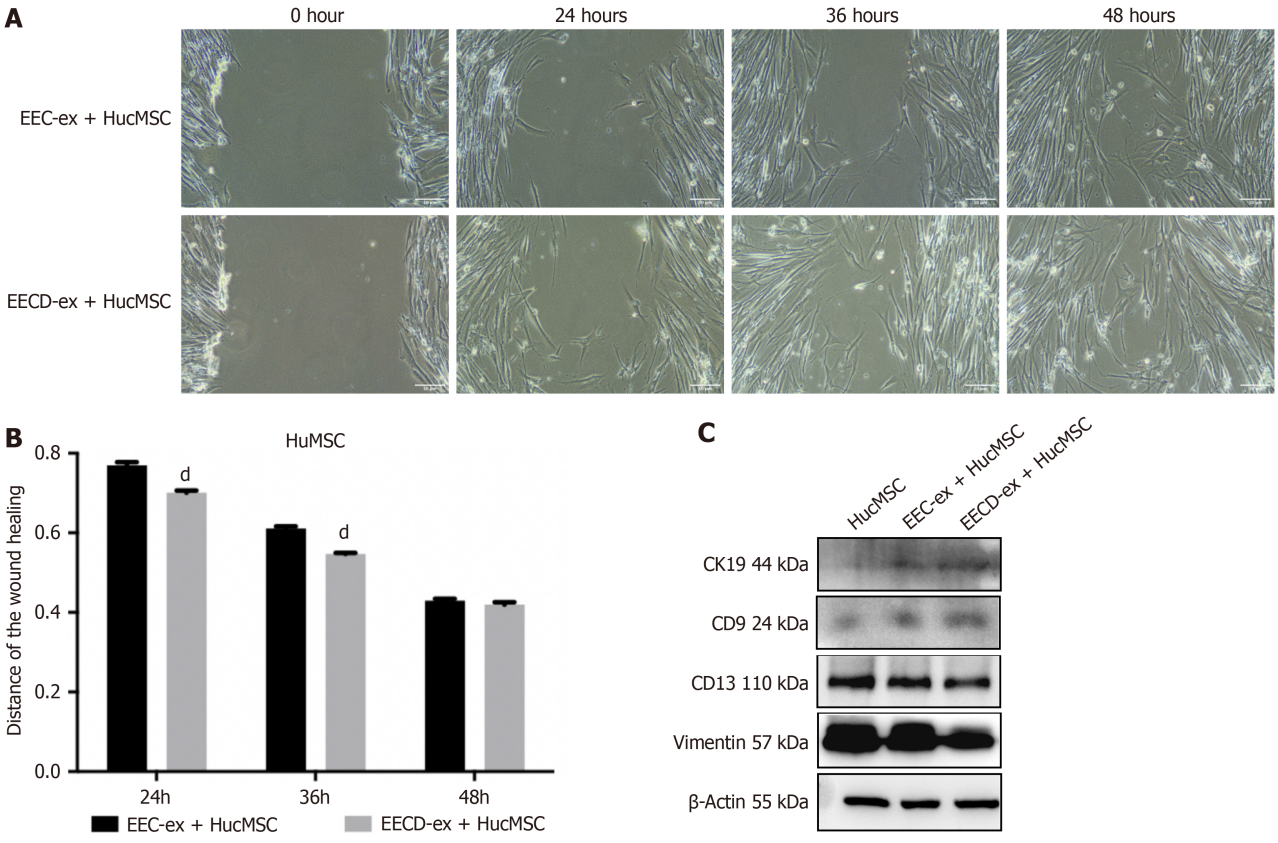


Figure 2 Exosomes derived from hypoxia-damaged endometrial epithelial cells promote human umbilical cord mesenchymal stem cell

migration and differentiation into endometrial epithelial cells. Human umbilical cord mesenchymal stem cells were cocultured with exosomes isolated from normal endometrial epithelial cells or exosomes derived from hypoxia-damaged endometrial epithelial cells. A and B: Human umbilical cord mesenchymal stem cell migration detected using wound healing assay; C: CD9, CK19, CD13, and vimentin protein levels determined by Western blotting. $n = 3$. $^{d}P < 0.0001$. Data are described as the mean ± SD. HucMSC: Human umbilical cord mesenchymal stem cells; EEC-ex: Exosomes isolated from normal endometrial epithelial cells; EECD-ex: Exosomes derived from hypoxia-damaged endometrial epithelial cells.

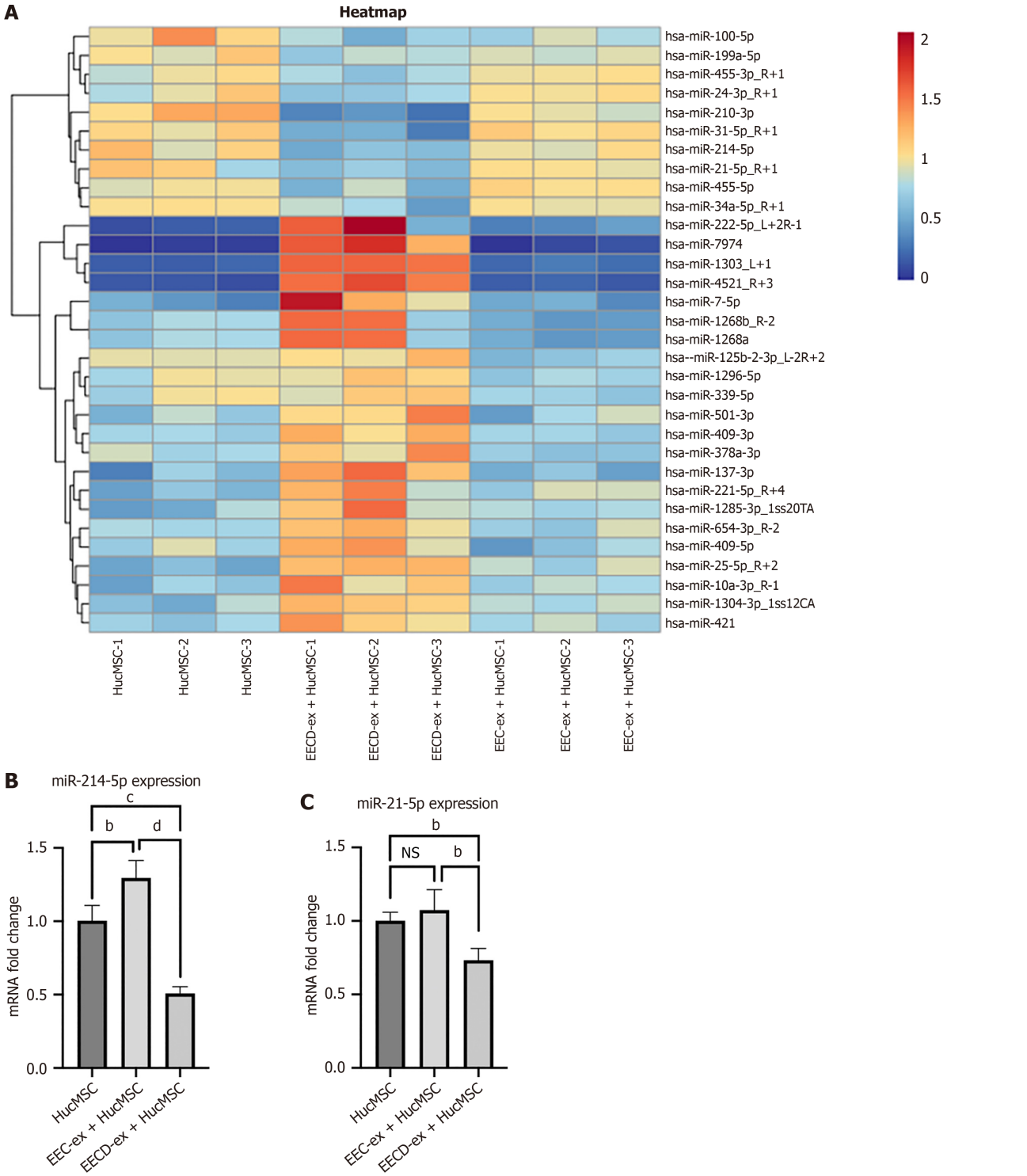
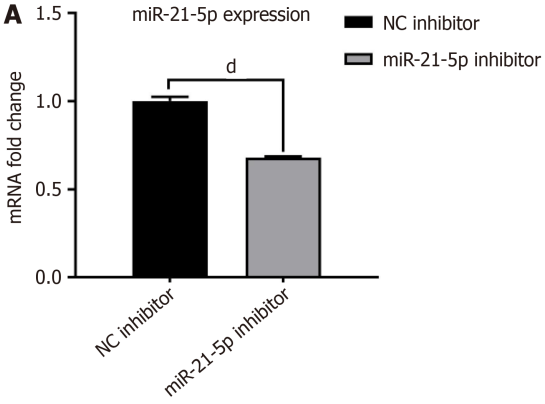
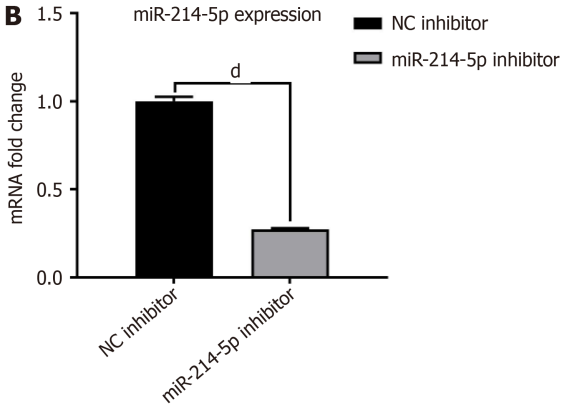
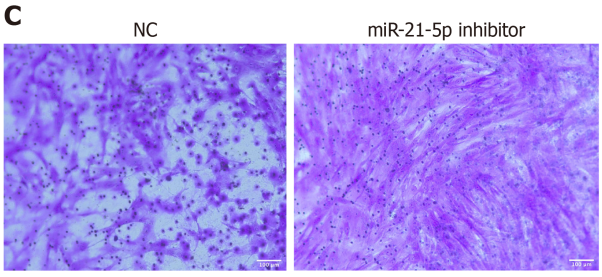


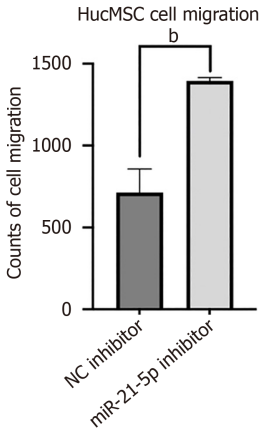
Figure 3 Differentially expressed microRNAs in human umbilical cord mesenchymal stem cells cocultured with exosomes derived from hypoxia-damaged endometrial epithelial cells. Human umbilical cord mesenchymal stem cells were cocultured with exosomes derived from endometrial epithelial cells or exosomes derived from hypoxia-damaged endometrial epithelial cells. A: Heat map from microRNA sequencing analysis showed microRNA profiles that were differentially expressed in human umbilical cord mesenchymal stem cells treated with exosomes derived from hypoxia-damaged endometrial epithelial cells or exosomes were isolated from normal endometrial epithelial cells; B and C: Reverse transcription-quantitative polymerase chain reaction for detecting miR-214-5p

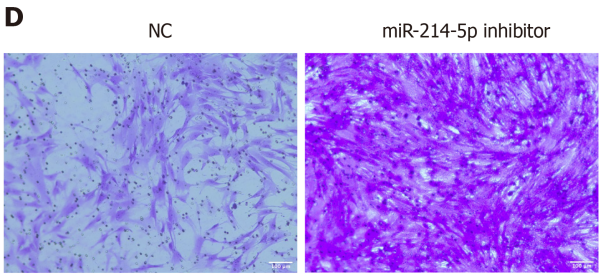
and miR-21-5p. n = 3. NS: No significant difference, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0001$. Data are described as the mean ± SD. HucMSC: Human umbilical cord mesenchymal stem cells; EEC-ex: Exosomes isolated from normal endometrial epithelial cells; EECD-ex: Exosomes derived from hypoxia-damaged endometrial epithelial cells.

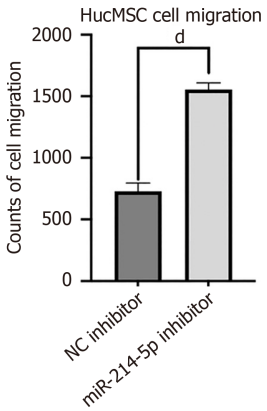


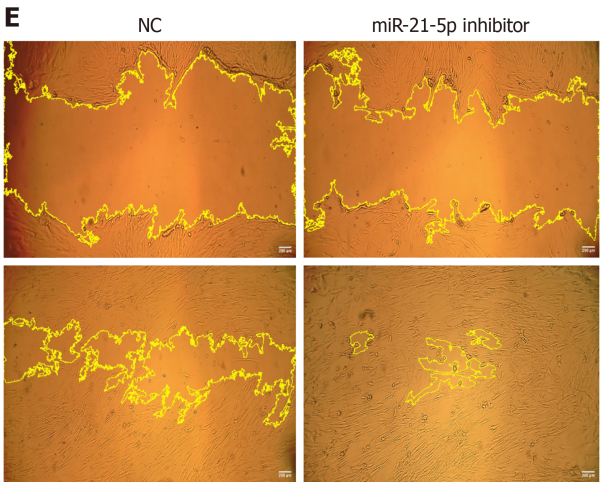


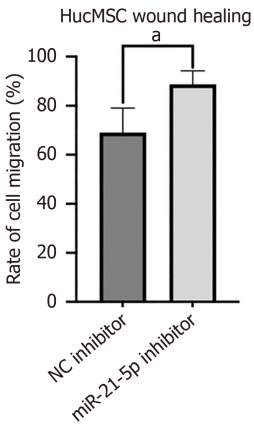


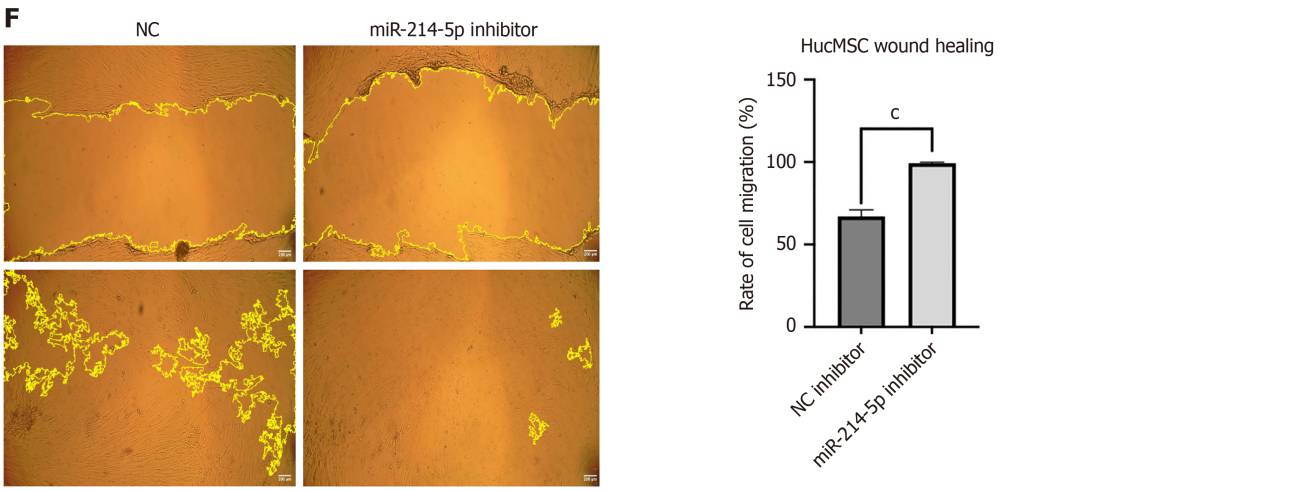











F
NC
miR-214-5p inhibitor
HucMSC wound healing
Rate of cell migration (%)
150
100
50
0
c
NC inhibitor
miR-214-5p inhibitor
G
24 hours
48 hours
72 hours
96 hours
NC
inhibitor
miR-21-5p
25 kDa
CD9
43 kDa
CK19
55 kDa
Vimentin
130 kDa
CD13
55 kDa
β-Tubulin
H
24 hours
48 hours
72 hours
96 hours
NC
inhibitor
miR-214-5p
25 kDa
CD9
43 kDa
CK19
55 kDa
Vimentin
130 kDa
CD13
55 kDa
β-Tubulin
I
CD9-FITC
DAPI
Merge
Ck19-PE
DAPI
Merge
NC
miR-21-5p inhibitor
CD13-FITC
DAPI
Merge
Vimentin-PE
DAPI
Merge
NC
miR-21-5p inhibitor

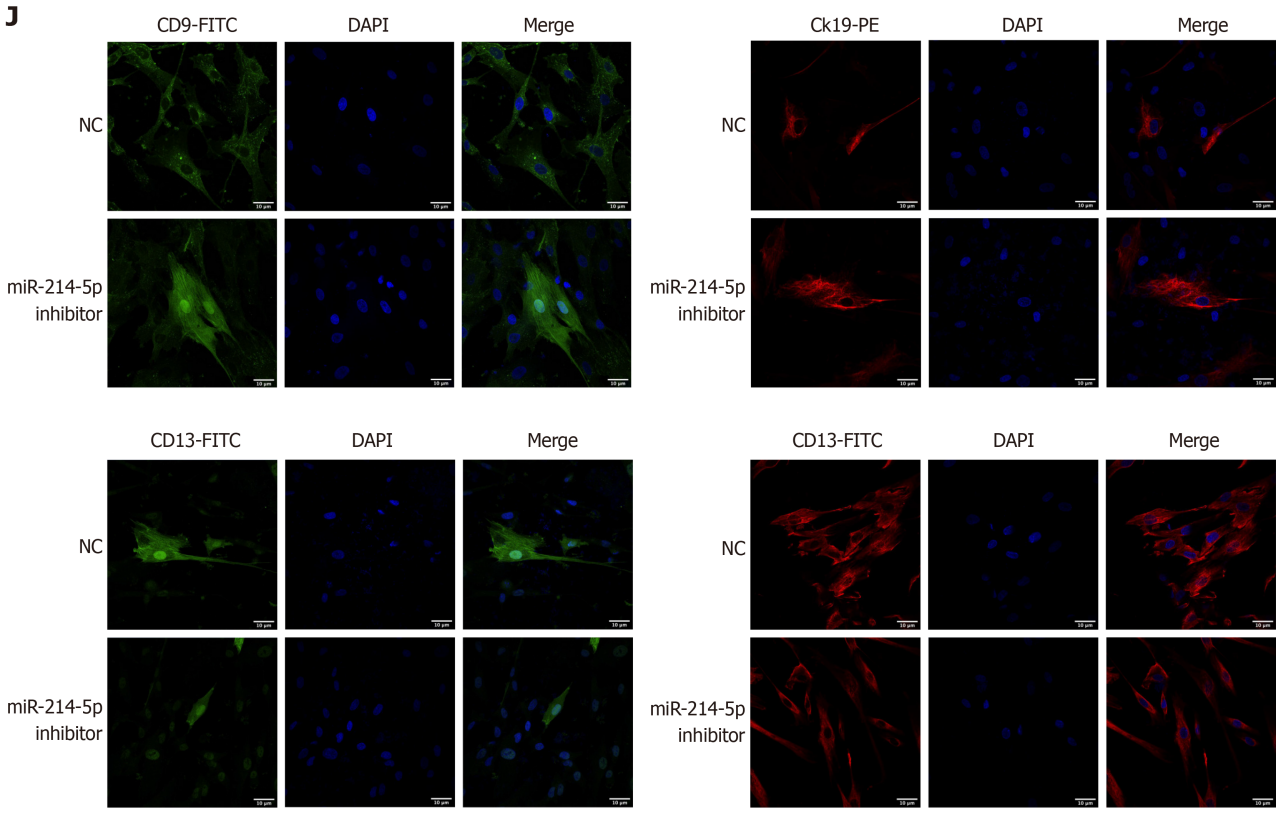


Figure 4 Silencing of miR-21-5p or miR-214-5p promotes human umbilical cord mesenchymal stem cell migration and differentiation into endometrial epithelial cells. MiR-21-5p/miR-214-5p inhibitor was transfected into human umbilical cord mesenchymal stem cells. A and B: MiR-21-5p and miR-214-5p levels measured by reverse transcription-quantitative polymerase chain reaction; C and D: Cell migration determined using Transwell assay; E and F: Wound healing assay for measurement of cell migration; G and H: Protein expression of CD9, CK19, vimentin, and CD13 measured using Western blotting; I and J: Immunofluorescence staining for detecting expression of CD9, CK19, CD13, and vimentin. $n = 3$. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0001$. Data are described as the mean ± SD. HucMSC: Human umbilical cord mesenchymal stem cells; NC: Normal control.

decreased (Supplementary Figure 2A-C and E-G). The five key regulatory genes related to osteogenic differentiation, alginate-like polymers, osteoprotegerin, bone morphogenetic protein 2, osteocalcin, and RunX2, were significantly decreased at both the gene transcription (RT-qPCR) (Supplementary Figure 2D and H) and protein expression (Western blotting) (Supplementary Figure 2I-L) levels. Oil Red O staining of the cells after induction of adipogenic differentiation showed that, compared with the transfection of the NC inhibitor control sequence, after transfection with miR-21-5p or miR-214-5p inhibitor, the size and proportion of lipid droplets in HucMSCs after induction of adipogenic differentiation were significantly decreased (Supplementary Figure 3A-D). The key regulatory genes of adipogenic differentiation, CCAAT/enhancer binding protein beta-α and peroxisome proliferator-activated receptor-γ, were significantly decreased at both the gene transcription (RT-qPCR) and protein expression (Western blotting) (Supplementary Figure 3E-J) levels. The above outcomes indicated that miR-21-5p and miR-214-5p inhibitors promoted HucMSC migration and differentiation into EECs.

miR-214-5p and miR-21-5p target PIAS3 and STAT3, respectively

miR-214-5p was uncovered to target *PIAS3*-3′-UTR and miR-21-5p targets *STAT3* 3′-UTR, while the binding of miRNAs to mRNA 3′-UTR loosened after 3′-UTR mutation, which was verified by dual-luciferase reporter assay (Figure 5A and B). Subsequently, the silencing efficiency of miR-214-5p and miR-21-5p inhibitors was assessed through RT-PCR. Compared to the NC inhibitor group, the RNA expression level of miR-214-5p in the miR-214-5p inhibitor group was markedly reduced. Similarly, the RNA expression level of miR-21-5p in the miR-21-5p inhibitor group was significantly diminished relative to the NC inhibitor group (Figure 5C and D). MiR-21-5p inhibitor upregulated *STAT3* mRNA level but downregulated *PIAS3* mRNA level in HucMSCs (Figure 5E and F). MiR-214-5p inhibitor upregulated *STAT3* and *PIAS3* mRNA levels in HucMSCs (Figure 5G and H). Additionally, phosphorylated (p)-STAT3 protein expression was elevated after miR-214-5p or miR-21-5p silencing in HucMSCs (Figure 5I).

STAT3 inhibitor reverses the effects induced by miR-214-5p or miR-21-5p silencing on HucMSC function

We explored whether miR-214-5p or miR-21-5p mediated the functional alterations of HucMSCs *via* regulation of STAT3. HucMSC transfected with miR-21-5p or miR-214-5p inhibitor were treated with the STAT3 inhibitor Stattic. Transwell assay showed that miR-214-5p or miR-21-5p inhibitor enhanced HucMSC migration, which was repressed after addition of the STAT3 inhibitor (Figure 6A and B). Wound healing assay also exhibited consistent results with those from the Transwell assay (Figure 6C and D). Compared with the NC inhibitor group, CD9 and CK19 were increased while vi-

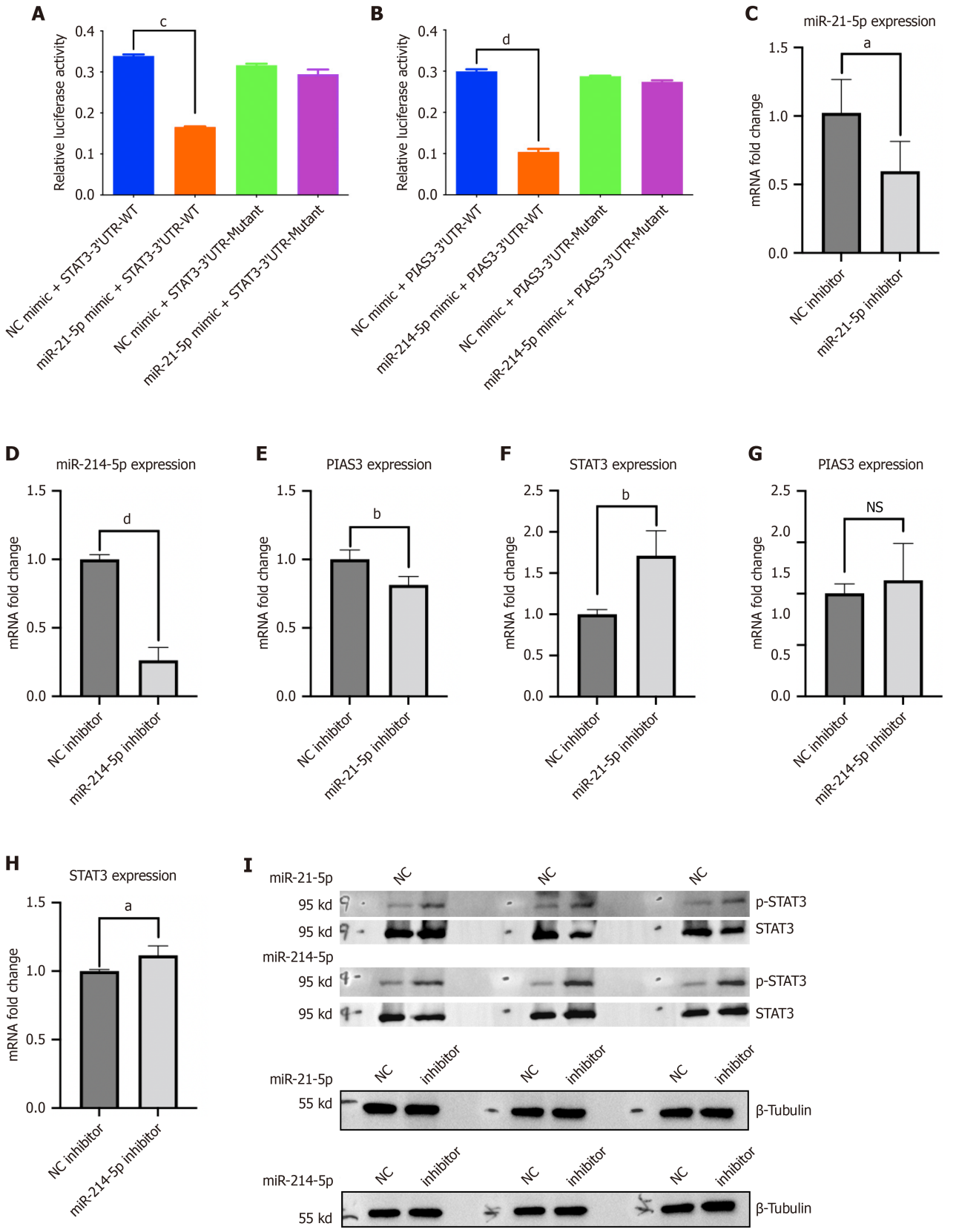
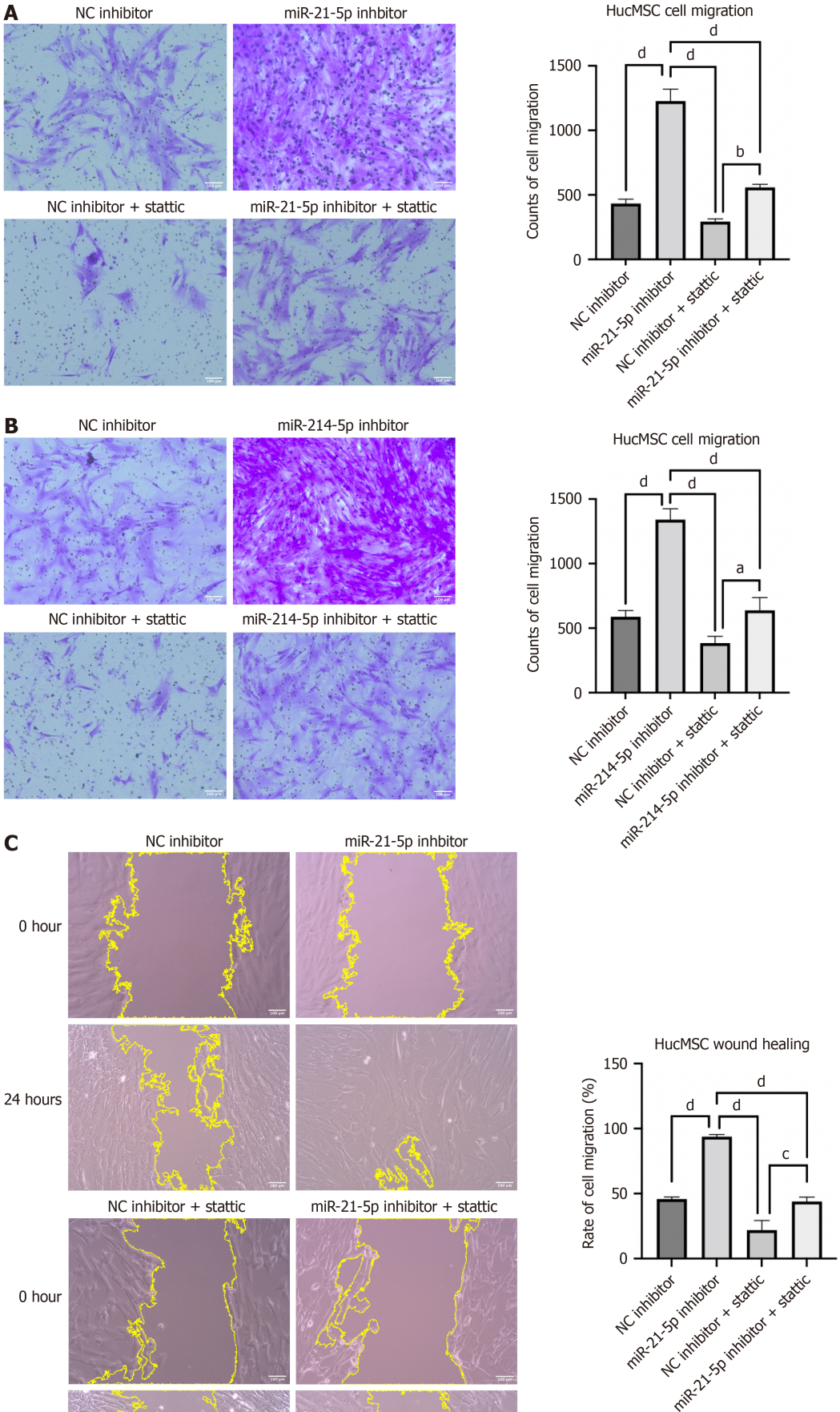
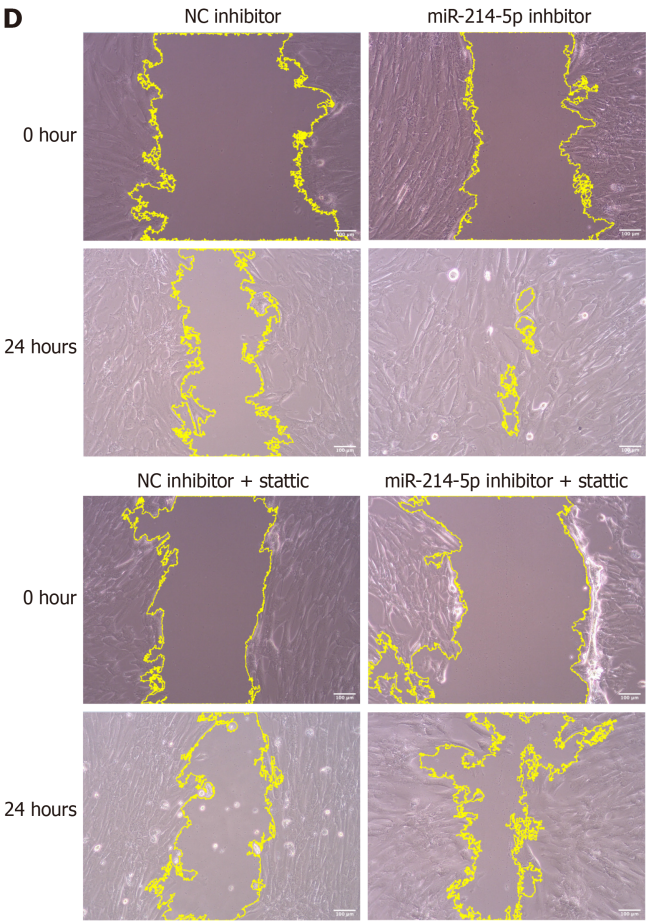
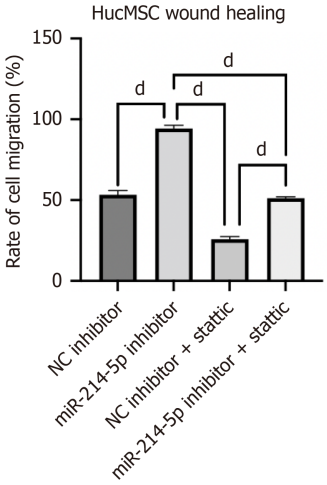
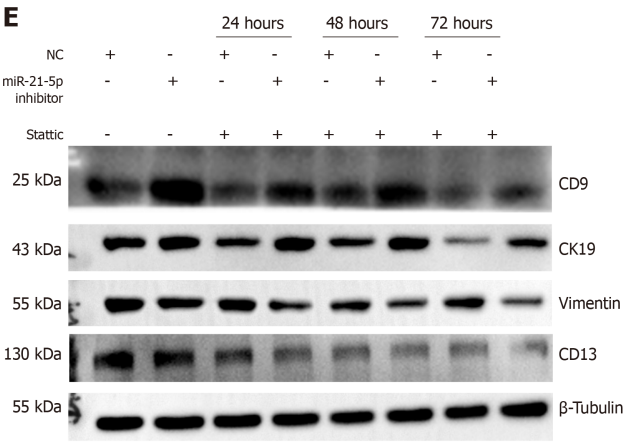


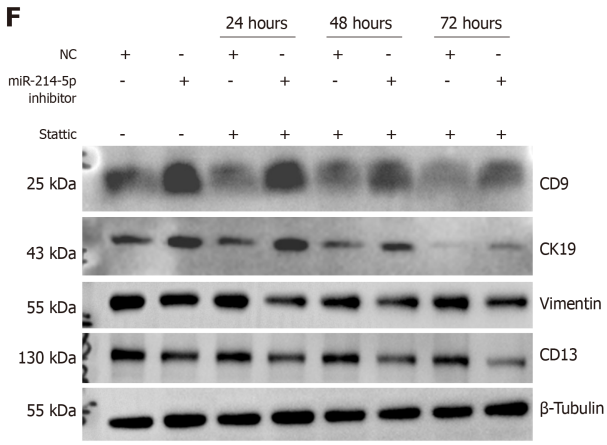
Figure 5 Signal transducer and activator of transcription 3 and protein inhibitor of activated signal transducer and activator of transcription 3 are the targets of miR-21-5p and miR-214-5p, respectively. A and B: Relative luciferase activities assessed by dual-luciferase reporter assay; C-H: MiR-21-5p, miR-214-5p, signal transducer and activator of transcription 3 (*STAT3*), and *PIAS3* mRNA expression detected by reverse transcription-quantitative polymerase chain reaction; I: Western blot analysis of STAT3 and phospho (p)-STAT3 protein levels. $n = 3$. NS: No significant difference, $^{a}P < 0.05$, $^{b}P < 0.01$, $^{d}P < 0.0001$. Data are described as the mean ± SD. STAT3: Signal transducer and activator of transcription 3; NC: Normal control.

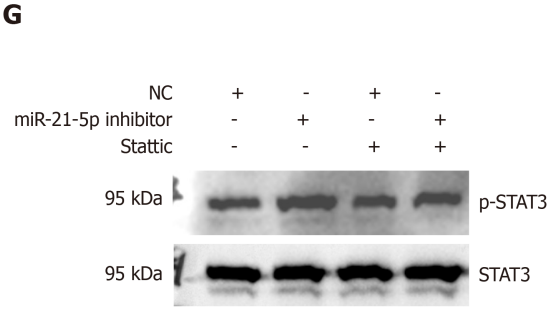

A
NC inhibitor
miR-21-5p inhbitor
NC inhibitor + stattic
miR-21-5p inhibitor + stattic
B
NC inhibitor
miR-214-5p inhbitor
NC inhibitor + stattic
miR-214-5p inhibitor + stattic
C
NC inhibitor
miR-21-5p inhbitor
0 hour
24 hours
NC inhibitor + stattic
miR-21-5p inhibitor + stattic
0 hour
24 hours
HucMSC cell migration
Counts of cell migration
1500
1000
500
0
d
d
d
b
NC inhibitor
miR-21-5p inhibitor
NC inhibitor + stattic
miR-21-5p inhibitor + stattic
HucMSC cell migration
Counts of cell migration
1500
1000
500
0
d
d
d
a
NC inhibitor
miR-214-5p inhibitor
NC inhibitor + stattic
miR-214-5p inhibitor + stattic
HucMSC wound healing
Rate of cell migration (%)
150
100
50
0
d
d
d
c
NC inhibitor
miR-21-5p inhibitor
NC inhibitor + stattic
miR-21-5p inhibitor + stattic

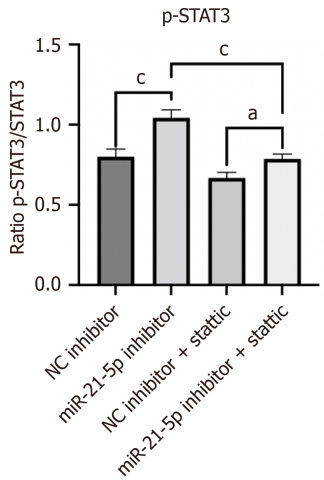

D
NC inhibitor
miR-214-5p inhbitor
0 hour
24 hours
NC inhibitor + stattic
miR-214-5p inhibitor + stattic
0 hour
24 hours


HucMSC wound healing
Rate of cell migration (%)
150
100
50
0
d
d
d
d
NC inhibitor
miR-214-5p inhibitor
NC inhibitor + stattic
miR-214-5p inhibitor + stattic


E
24 hours
48 hours
72 hours
NC
miR-21-5p inhibitor
Stattic
25 kDa
CD9
43 kDa
CK19
55 kDa
Vimentin
130 kDa
CD13
55 kDa
β-Tubulin


F
24 hours
48 hours
72 hours
NC
miR-214-5p inhibitor
Stattic
25 kDa
CD9
43 kDa
CK19
55 kDa
Vimentin
130 kDa
CD13
55 kDa
β-Tubulin


G
NC
miR-21-5p inhibitor
Stattic
95 kDa
p-STAT3
95 kDa
STAT3


p-STAT3
Ratio p-STAT3/STAT3
1.5
1.0
0.5
0.0
c
c
a
NC inhibitor
miR-21-5p inhibitor
NC inhibitor + stattic
miR-21-5p inhibitor + stattic

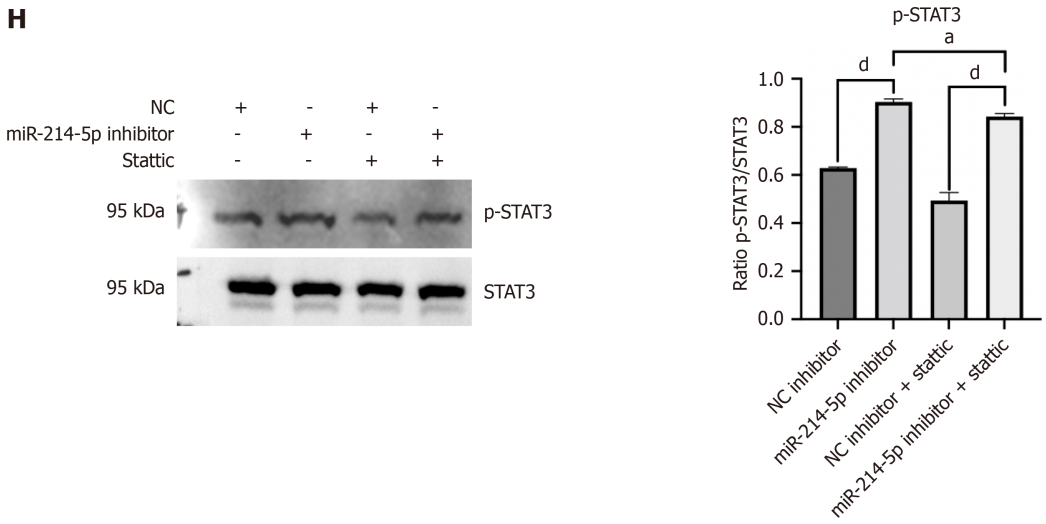


Figure 6 Signal transducer and activator of transcription 3 inhibitor reverses the effects induced by miR-21-5p or miR-214-5p inhibitor on human umbilical cord mesenchymal stem cell function. Human umbilical cord mesenchymal stem cells transfected with miR-21-5p or miR-214-5p inhibitor, and treated with signal transducer and activator of transcription 3 inhibitor (Stattic). A and B: Transwell assay for detecting human umbilical cord mesenchymal stem cells migration; C and D: Wound healing assay for detection of cell migration; E and F: Protein levels of CD9, CK19, vimentin, and CD13 determined by Western blotting; G and H: Western blotting for detecting signal transducer and activator of transcription 3 and phospho (p)-signal transducer and activator of transcription 3 protein levels. $n = 3$. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0001$. Data are described as the mean ± SD. HucMSC: Human umbilical cord mesenchymal stem cells; NC: Normal control; STAT3: Signal transducer and activator of transcription 3.

mentin and CD13 were decreased in HucMSCs after treatment with miR-214-5p or miR-21-5p inhibitor. However, after addition of the STAT3 inhibitor, CD9 and CK19 were gradually decreased, while vimentin and CD13 were also gradually decreased with the increase of treatment duration (Figure 6E and F). In addition, p-STAT3 was increased in HucMSCs after treatment with miR-214-5p or miR-21-5p inhibitor, while addition of the STAT3 inhibitor reduced p-STAT3 level (Figure 6G and H). The results suggested that inhibition of STAT3 reversed the effects induced by miR-21-5p or miR-214-5p inhibitor on HucMSC migration and differentiation.

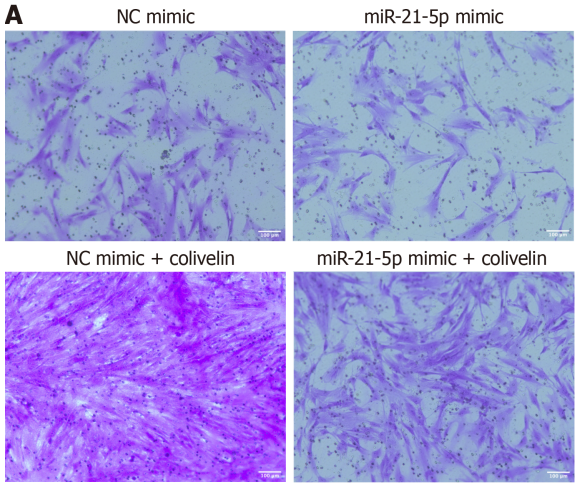
miR-21-5p or miR-214-5p overexpression represses HucMSC migration and differentiation into EEC, while STAT3 activator reverses these effects

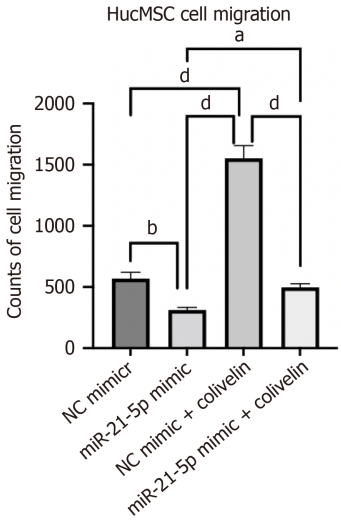
Transwell assay showed that miR-214-5p or miR-21-3p overexpression inhibited HucMSC migration compared with the NC mimic group, whereas addition of the STAT3 activator colivelin enhanced the migration of HucMSCs (Figure 7A and B). Wound healing assay showed similar results to the Transwell assay (Figure 7C and D). Compared with the NC mimic group, no obvious alteration was observed in the expression of CD9, CK19, vimentin, CD13, and p-STAT3 in HucMSCs after miR-21-5p or miR-214-5p overexpression. In contrast, CD9 and CK19 were increased, vimentin and CD13 were decreased, and p-STAT3 was increased in HucMSC treated with the STAT3 activator. Compared with the miR-21-5p or miR-214-5p mimic group, CD9 and CK19 increased, vimentin and CD13 decreased, and p-STAT3 increased in the miR-21-5p mimic + STAT3 activator or miR-214-5p mimic + STAT3 activator group (Figure 7E and F). Activation of STAT3 reversed the effects induced by miR-214-5p or miR-21-5p overexpression on HucMSC function.

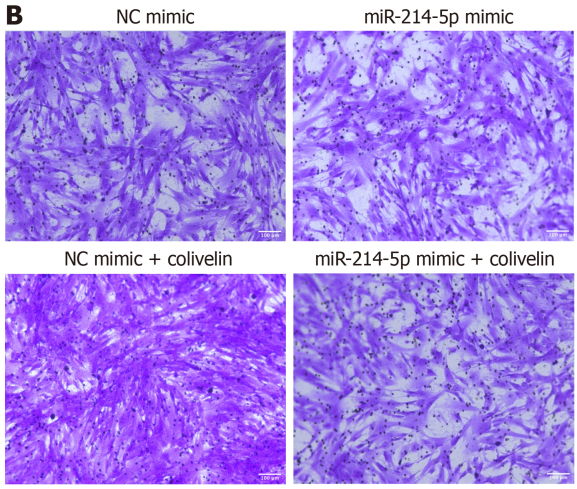
DISCUSSION

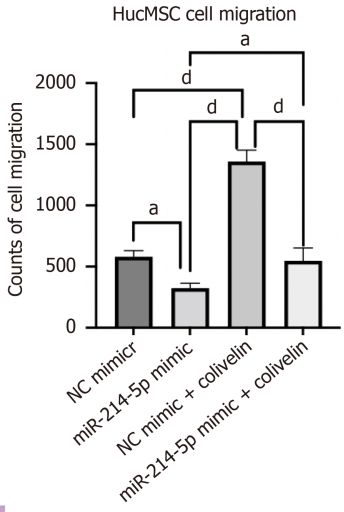
The management of thin endometrium for patients undergoing assisted reproduction is a common challenge[3]. A previous study has demonstrated that HucMSC-derived exosomes repair EEC damage caused by hypoxia[18]. Here, we found that exosomes derived from hypoxic EECs promoted HucMSC migration and differentiation into EECs. MiR-21-5p and miR-214-5p were lowly expressed in EECD-ex-pretreated HucMSCs. MiR-21-5p or miR-214-5p silencing promoted HucMSC migration and EEC differentiation *via* upregulating p-STAT3.

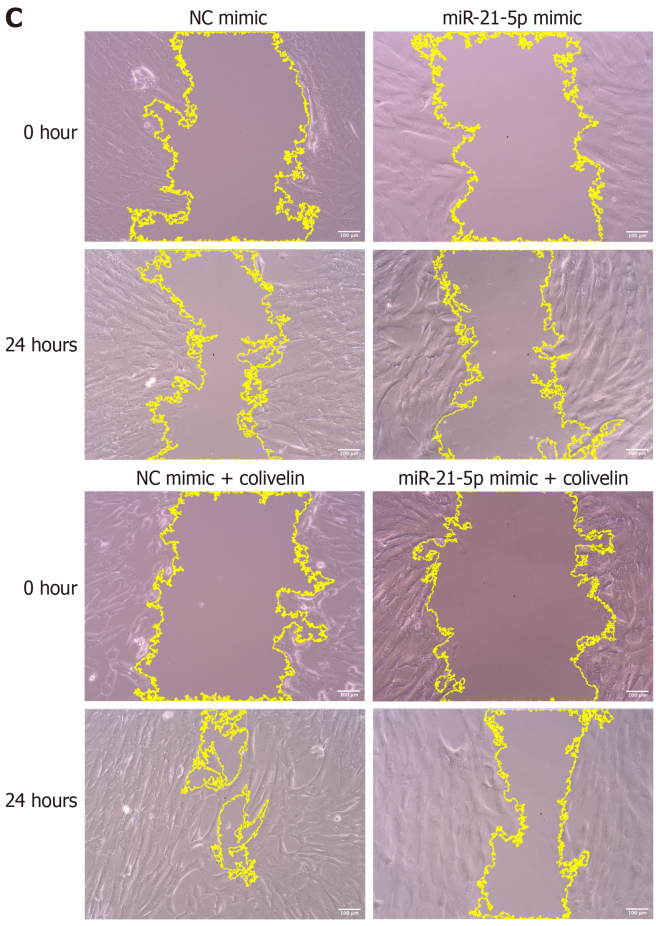
EEC-derived exosomes suppress cell migration and invasion in ovarian endometriosis[9]. Exosomes from bovine EECs ensured trophoblast cell development[10]. Exosomes released from ectopic endometrial cells induce the migration of eutopic endometrial cells[20]. In the present study, coculture with the exosomes derived from hypoxic EECs promoted the migration of HucMSCs. A study has uncovered the capacity of MSCs derived from human umbilical cord Wharton's jelly to differentiate into EEC-like cells in specific microenvironments[19]. We found that EECD-ex coculture promoted HucMSC differentiation into EECs, as revealed by the elevated CD9 and CK19 and reduced vimentin and CD13. The findings indicated that exosomes secreted from EECs acted as drivers of HucMSC migration and differentiation. We propose that EECD-exs might carry genetic information into HucMSCs, which promotes HucMSC migration and differentiation into EECs.

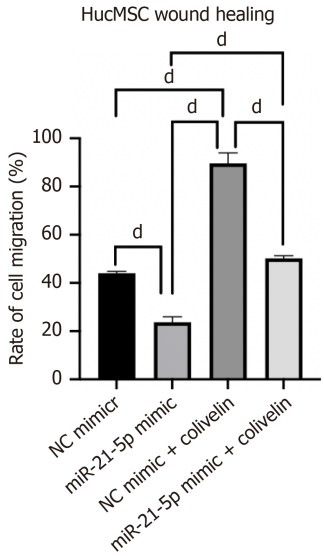

A
NC mimic
miR-21-5p mimic
NC mimic + colivelin
miR-21-5p mimic + colivelin

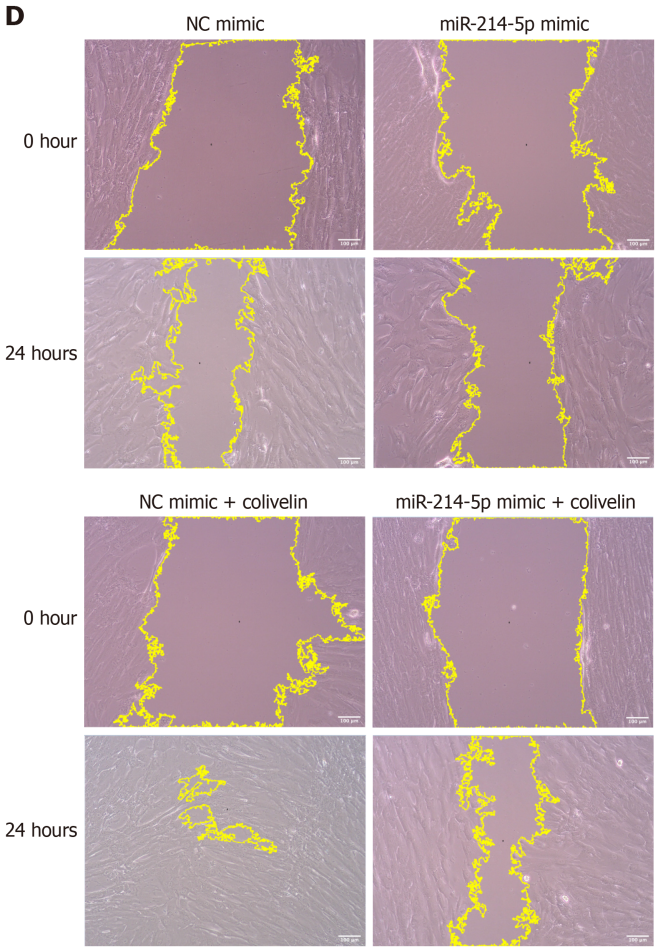

HucMSC cell migration
Counts of cell migration
2000
1500
1000
500
0
a
d
d
d
b
NC mimicr
miR-21-5p mimic
NC mimic + colivelin
miR-21-5p mimic + colivelin

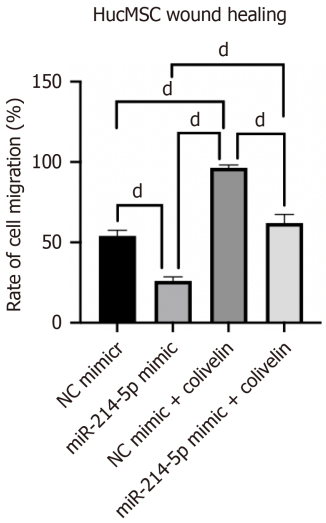

B
NC mimic
miR-214-5p mimic
NC mimic + colivelin
miR-214-5p mimic + colivelin

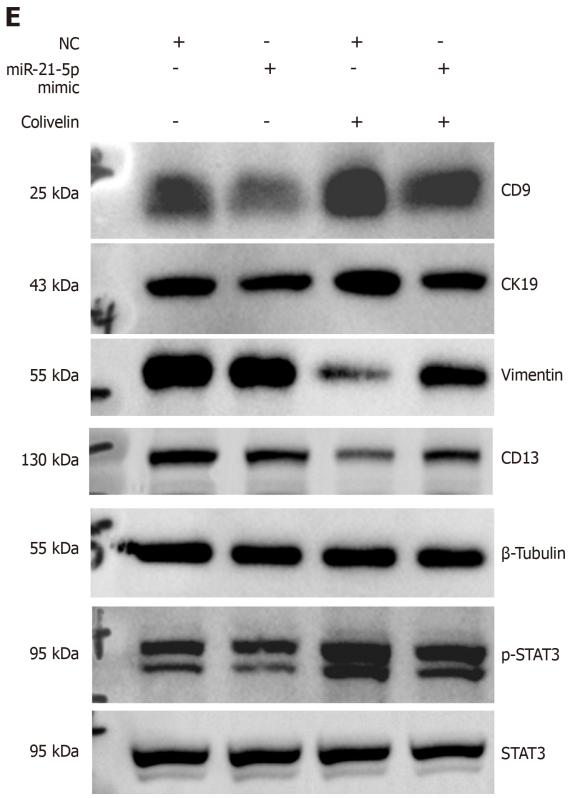

HucMSC cell migration
Counts of cell migration
2000
1500
1000
500
0
a
d
d
d
a
NC mimicr
miR-214-5p mimic
NC mimic + colivelin
miR-214-5p mimic + colivelin

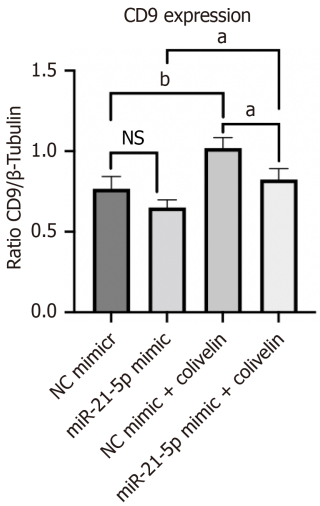

C
NC mimic
miR-21-5p mimic
0 hour
24 hours
NC mimic + colivelin
miR-21-5p mimic + colivelin
0 hour
24 hours

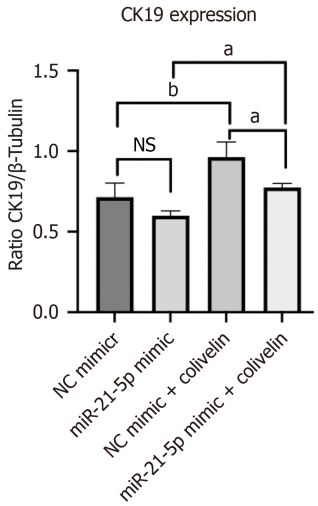

HucMSC wound healing
Rate of cell migration (%)
100
80
60
40
20
0
d
d
d
d
d
NC mimicr
miR-21-5p mimic
NC mimic + colivelin
miR-21-5p mimic + colivelin


D
NC mimic
miR-214-5p mimic
0 hour
24 hours
NC mimic + colivelin
miR-214-5p mimic + colivelin
0 hour
24 hours


HucMSC wound healing
Rate of cell migration (%)
150
100
50
0
d
d
d
d
d
NC mimicr
miR-214-5p mimic
NC mimic + colivelin
miR-214-5p mimic + colivelin


E
NC
miR-21-5p mimic
Colivelin
25 kDa
CD9
43 kDa
CK19
55 kDa
Vimentin
130 kDa
CD13
55 kDa
β-Tubulin
95 kDa
p-STAT3
95 kDa
STAT3


CD9 expression
Ratio CD9/β-Tubulin
1.5
1.0
0.5
0.0
a
b
a
NS
NC mimicr
miR-21-5p mimic
NC mimic + colivelin
miR-21-5p mimic + colivelin


CK19 expression
Ratio CK19/β-Tubulin
1.5
1.0
0.5
0.0
a
b
a
NS
NC mimicr
miR-21-5p mimic
NC mimic + colivelin
miR-21-5p mimic + colivelin

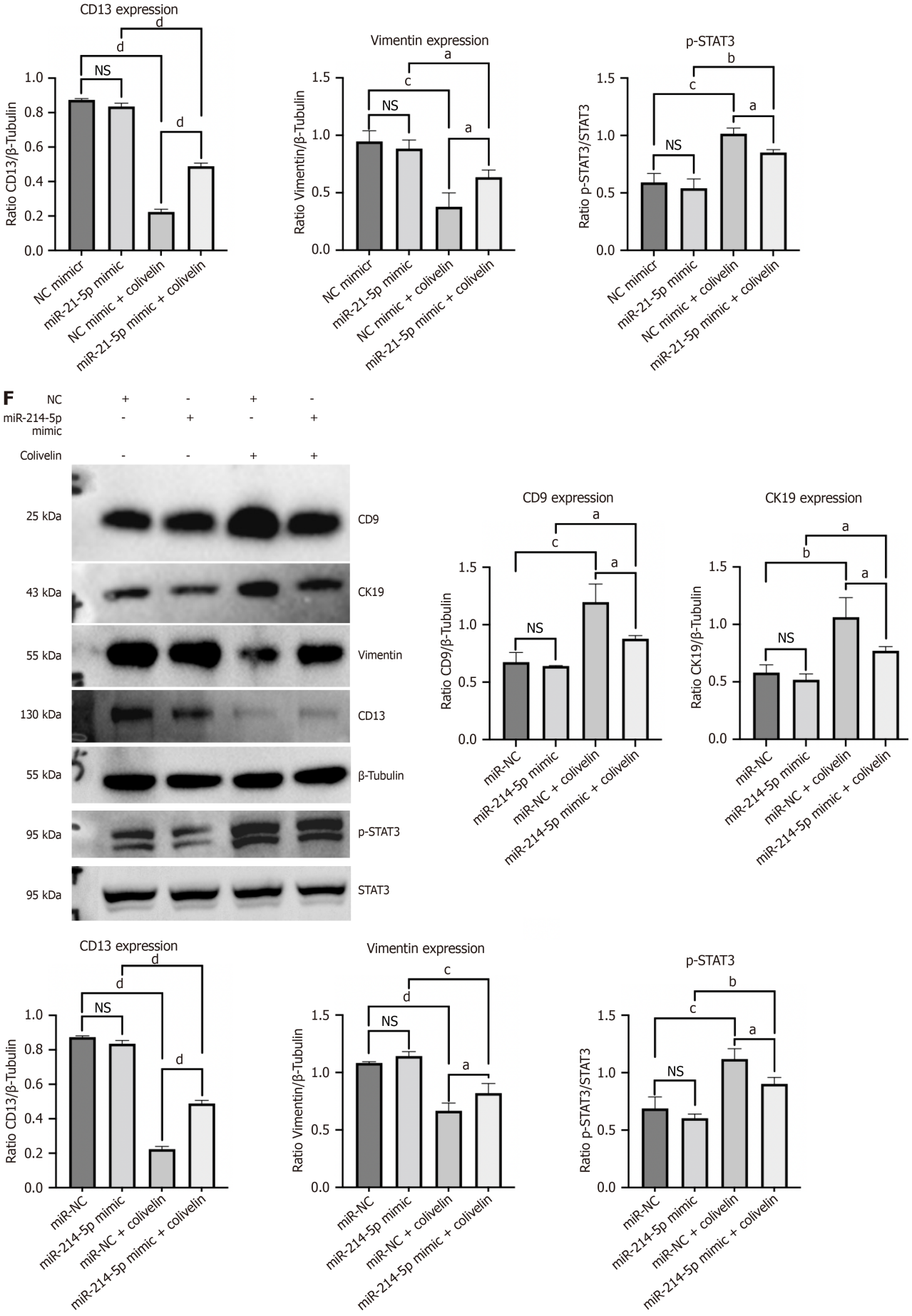


Figure 7 MiR-21-5p or miR-214-5p overexpression represses human umbilical cord mesenchymal stem cell migration and differentiation

into endometrial epithelial cells, while signal transducer and activator of transcription 3 activator reverses these effects. Human umbilical cord mesenchymal stem cells were transfected with miR-214-5p or miR21-3p mimic, and treated with signal transducer and activator of transcription 3 activator (colivelin). A and B: Transwell assay for detecting human umbilical cord mesenchymal stem cell migration; C and D: Wound healing assay for measurement of cell migration; E and F: Western blotting for measuring CD9, CK19, vimentin, CD13, and phospho (p)-signal transducer and activator of transcription 3. $n = 3$. NS: No significant difference, $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0001$. Data are described as the mean ± SD. HucMSC: Human umbilical cord mesenchymal stem cells; NC: Normal control; STAT3: Signal transducer and activator of transcription 3.

MiR-214-3p is reduced in endometrial cancer cells and tissues[21], and ectopic lesions and stromal cells in endometriosis[22]. MiR-214 level is reduced in ectopic ESCs[23]. Patients with intrauterine adhesion have a decrease in miR-21-5p in the endometrium[24]. In the present study, differentially expressed miRNAs were screened by miRNA sequencing analysis, and confirmed by RT-qPCR. We observed a low level of miR-214-5p and miR-21-5p in EECD-ex-cocultured HucMSCs. MiR-21-5p is implicated in modulating EEC proliferation and migration[25], and miR-21-5p[26,27] and miR-214-5p[28,29] are involved in regulation of osteogenic differentiation in mesenchymal stromal cells. Importantly, miR-21-5p or miR-214-5p silencing promoted HucMSC migration and differentiation into EECs, whereas miR-214-5p or miR-21-5p overexpression had opposite effects. The present study is believed to be the first to reveal the biological function of miR-214-5p and miR-21-5p in regulating differentiation of HucMSCs into EECs.

Hypoxia in endometriotic lesions induces aberrant activation of the STAT3 signaling pathway[30]. In humans, p-STAT3 is highly expressed in the endometrium from endometriosis patients and in adenomyosis lesions[31,32]. PIAS3 is a negative regulator of STAT3. PIAS3 represses STAT3 transcriptional activity by binding to the STAT3 DNA-binding domain[33]. Attenuation of PIAS3 increases p-STAT3 levels, causing aberrant STAT3 activation in endometriosis[34]. miRDB and TargetScan predicted that PIAS3 and STAT3 are the latent targets of miR-214-5p and miR-21-5p, respectively. A previous study has verified the binding of miR-21-5p to STAT3, and that miR-21-5p negatively regulates STAT3 expression[35,36]. We also verified the direct relationship between STAT3 and miR-21-5p. The direct interaction between PIAS3 and miR-214-5p was also validated. MiR-214-5p and miR-21-5p inhibitors downregulated *PIAS3* mRNA and upregulated *STAT3* mRNA and p-STAT3 protein in HucMSCs. A previous study has revealed that STAT3 is activated in myofibroblast differentiation of endometrial MSCs[37]. Importantly, inhibition of STAT3 reversed the effects induced by miR-21-5p or miR-214-5p inhibitor on HucMSC function. Functional assays using STAT3 activator and miR-21-5p/miR-214-5p mimics also displayed consistent outcomes. We propose that miR-21-5p or miR-214-5p inactivates the STAT3 signaling pathway to regulate the function of HucMSCs.

CONCLUSION

This research demonstrated that lowly expressed miR-214-5p and miR-21-5p in hypoxic-EEC-derived exosomes might activate the STAT3 signaling pathway to promote HucMSC migration and differentiation into EECs. Therapeutics to target and regulate miR-214-5p and miR-21-5p expression may be helpful in treating thin endometrium.

FOOTNOTES

Author contributions: Deng CY conceptualized and designed the study; Wang HB drafted the initial manuscript; Zhang WY collected the data and carried out the initial analyses. Deng CY and Wang HB contribute equally to the study. All authors critically reviewed the manuscript for important intellectual content, approved the final manuscript as submitted, and agree to be accountable for work.

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