Decidual small extracellular vesicles induce trophoblast invasion by upregulating N-cadherin

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Abstract

Small extracellular vesicles (sEVs) are important mediators of cell-to-cell communication involved in the successful establishment of a pregnancy. Human decidual stromal cells play a key role in regulating trophoblast invasion. Nevertheless, the regulatory functions of decidual stromal cells-derived sEVs in human trophoblast cells are still unclear. In this study, primary human decidual stromal cells were isolated, and immortalized human endometrial stromal cell line (HESCs) were decidualized into human decidual stromal cells (HDSCs) using hormonal cocktail containing medroxy progesterone 17-acetate (MPA), estrogen and cAMP analog. HDSC-sEVs were isolated from both primary human decidual stromal cells and immortal HDSCs, respectively, and identified by transmission electron microscopy and western blotting. EV uptake assay indicated that HDSC-sEVs could be uptaken by trophoblast cells. HDSC-sEVs could increase the invasiveness and the expression level of N-cadherin of trophoblast cells with elevated phosphorylation of SMAD2 and SMAD3 in the cells. Silencing of N-cadherin could block cell invasion induced by HDSC-sEVs, while knockdown of SMAD2 and SMAD3 could inhibit the upregulation of N-cadherin in trophoblast cells. Taken together, our results suggested a regulatory effect of HDSC-sEVs in the invasion of trophoblast cells, and HDSC-sEVs may be important mediators of trophoblasts during embryo implantation and placentation.

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Introduction

Successful pregnancy depends on the normal process of endometrial decidualization, embryo implantation, and placentation. Each step is indispensable to the next (Carson *et al.* 2000, Dey *et al.* 2004). Trophoblasts are specialized cells of placenta that play a major role in the implantation and formation of the maternal–fetal interface. Unlike uncontrolled invasion observed in tumors, trophoblastic invasion is a highly regulated process (Red-Horse *et al.* 2004). Inappropriate trophoblast invasion is implicated in several pregnancy-related disorders, such as intrauterine growth restriction and preeclampsia (PE) (Brosens *et al.* 1967, Pijnenborg *et al.* 1991, Sankaralingam *et al.* 2006). It is important to unravel the molecular mechanisms in regulating trophoblast invasion.

Endometrium undergoes decidualization before the invasion of trophoblast. The significant change occurred during decidualization is the transformation of endometrial stromal cells into highly specialized decidual stromal cells that exhibit a distinct function (Gellersen and Brosens 2014, Sharma *et al.* 2016). The cross-talk between trophoblast and decidual stromal

cells plays a key role in the appropriate invasion of trophoblast cells (Sharma *et al.* 2016). Decidual stromal cells could secrete many pro- and anti-invasion factors that may balance the invasiveness of trophoblast cells (Godbole *et al.* 2011). However, the mechanisms of how decidual stromal cells regulate trophoblastic invasion are not fully understood and further investigation may lead to a deeper understanding of the pathology of placenta-related complications.

Extracellular vesicles (EVs) have recently evoked considerable interest among researchers as these are released by many types of cells containing different cargoes, such as microRNA, mRNA and chemokines, which play important roles in cell-to-cell communication. Small EVs (sEVs) represent a particular subtype of extracellular vesicles with the diameter of 40–300 nm and were reported to be involved in regulating the activity of both neighboring and distant recipient cells through their signaling molecules (Maas *et al.* 2017, Théry *et al.* 2018). It has been reported that sEVs derived from either embryo or endometrium are involved in endometrial receptivity and may reflect placental health status (Homer *et al.* 2017, Tannetta *et al.* 2017). sEVs could promote the invasion of many

types of cancer cells through epithelial–mesenchymal transition (EMT) by upregulating the expression level of N-cadherin (Gopal *et al.* 2017). N-cadherin is one of the cell adhesion molecules that belongs to the cadherin subclasses and is a hallmark of EMT (Hansen *et al.* 2008, Mrozik *et al.* 2018). During implantation, trophoblast cells exhibit invasive ability akin to that of cancer cells and N-cadherin is involved in this process (Zhao *et al.* 2018).

This study aimed to determine the role of sEVs derived from human decidual stromal cells in regulating trophoblast function and to identify the molecular mechanisms involved in this process. Our research demonstrated that human decidual stromal cells-derived sEVs (HDSC-sEVs) could induce invasiveness of trophoblast cells and that this effect was induced via the upregulation of N-cadherin through SMAD2/3 signaling pathway. These findings might provide further evidence to support the key role of human decidual stromal cells in modulating the invasion of trophoblasts in a controlled manner required for a successful pregnancy.

Materials and methods

Ethics

Thirty first-trimester human placental specimens (gestational age: 6–12 weeks) were obtained from women undergoing legal elective termination of pregnancy. This study was approved by Nanfang Hospital, Southern Medical University (Study number: NFEC-2017-055). All procedures were performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all pregnant women involved in this study.

Tissue collection and isolation of primary trophoblast cells

The methodology for the isolation of extravillous trophoblast (EVT) cells from first-trimester human placenta was described elsewhere (Li *et al.* 2018). Briefly, placental tissues (6–12 weeks, $n=15$) were washed three times with cold sterile Dulbecco's PBS (Gibco, Invitrogen) and digested with trypsin (Gibco) and Dispase/DNAse (Gibco) with agitation at 300 rpm for 20 min at 37°C. The remaining tissues were sent for another three cycles of digestion. Fetal bovine serum [20% (v/v) (FBS, PAN-Biotech)] was added to terminate the digestion. All the tissues were collected and layered using Percoll (Gibco) gradient [20% (v/v), 40% (v/v), and 60% (v/v)] centrifugation at 994 *g* for 20 min at 4°C. The primary trophoblast cells were collected from the 40% (v/v)/60% (v/v) interfaces. Cells were resuspended in PBS and centrifuged at 300 *g* for 10 min at 4°C. Subsequently, the cells were plated in tissue culture flasks in RPMI 1640 Medium (Gibco) with 20% FBS, streptomycin (100 mg/mL) and penicillin (100 U/mL) at 37°C in a humidified 5% CO₂/air atmosphere.

Immunofluorescence analyses of primary human EVTs

Primary human EVTs (1.5×10^4) were seeded onto the 15 mm coverslips and allowed to attach for 24 h (37°C, 5% CO₂) in RMPI 1640 with 20% FBS. Cells were then fixed with 4% paraformaldehyde for 10 min and washed twice with glutaraldehyde. Immunofluorescence staining was performed as previously described (Johnson *et al.* 2001). After being washed with PBS containing 0.3% vol/vol Tween 20, cells were blocked with 10% vol/vol goat serum and then incubated overnight at 4°C with a MAB to human cytokeratin 7 (1 mg/mL, Abcam, AB9021). Cell-bound primary antibody was then detected using goat anti-mouse IgG Alexa 488 (1 mg/mL, Life Technologies). Coverslips were adhered to corning pre-cleaned single-frosted slides via Prolong Antifade Mounting Reagent with DAPI (Invitrogen).

Isolation of primary human DSCs and treatments

Human decidua tissues were obtained following legal surgically terminated pregnancies at the Department of Obstetrics and Gynecology, Nanfang Hospital. Primary human decidual stromal cells were isolated according to the previously reported methods (Li *et al.* 2010). In brief, decidua tissues were obtained following elective terminations of pregnancy ($n=15$), washed in PBS three times and minced into small pieces in phenol red-free DMEM/F12 in which tissues were digested with 0.1% collagenase and 0.1 mg/mL DNase for 1 h at 37°C. The enzymatic reaction was terminated by adding phenol red-free DMEM/F12 medium with 20% (vol/vol) fetal calf serum (Gibco). The suspension was filtered through a sterile 100 μ m pore gauze mesh centrifuged at 400 *g* for 15 min. Cell pellet was suspended in phenol red-free DMEM/F12 and centrifuged using a discontinuous Percoll (Sigma) gradient of 20, 40 and 60% (vol/vol) at 994 *g* for 20 min. The human DSCs were collected from the 20–40% interface and then suspended in phenol red-free DMEM/F12 medium with 20% (vol/vol) FCS and incubated in cell plate. After cultured for 45 min, the non-adherent lymphocytes were removed.

After the isolation of human DSCs, we cultured primary human DSCs in phenol red-free DMEM/F12 (Gibco) containing 10% (v/v) charcoal-stripped fetal bovine serum (CS-FBS, PAN-Biotech, Bavaria, Germany), 3.1 g/L glucose (Sigma-Aldrich) and 1 mM sodium pyruvate (Sigma-Aldrich) supplemented with 50 mg/mL penicillin–streptomycin (Gibco), 1.5 g/L sodium bicarbonate (Sigma-Aldrich), 500 ng/mL puromycin (Sigma-Aldrich) and 1% insulin–transferrin–selenium (Gibco). The primary human DSCs were passaged three to four times before *in vitro* treatments. We re-decidualized primary human DSCs with medium containing 1% CS-FBS, 10^{-8} M estrogen (E, Sigma-Aldrich) plus 10^{-7} M medroxyprogesterone-17-acetate (MPA, Abcam) and 0.5 mM dibutyl cAMP (db-cAMP, Sigma-Aldrich) for 8 days as previously reported (James-Allan *et al.* 2018). Primary HDSCs were cultured using DSC culture media with 10% EVs-depleted FCS, 10^{-8} M E plus 10^{-7} M MPA and 0.5 mM db-cAMP for 48 h and the conditioned medium was collected to isolate primary HDSC-sEVs. The EVs-depleted FCS was prepared as previously reported (Yan *et al.* 2018). In brief, we prepared EVs-depleted FBS by overnight ultracentrifugation of FCS at 100,000 *g* at 4 °C.

Immunofluorescence analyses of primary human DSCs

Primary human DSCs (1.5×10^4) were seeded onto the 15 mm coverslips and allowed to attach for 24 h (37°C, 5% CO₂) in phenol red-free with 20% FCS. Cells were then fixed with 4% paraformaldehyde for 10 min and washed twice with glutaraldehyde. Immunofluorescence staining was performed as previously described (Johnson *et al.* 2001). After being washed using PBS containing 0.3% vol/vol Tween 20, cells were blocked with 10% (vol/vol) goat serum followed by incubation overnight at 4°C with a MAB to human vimentin (1 mg/mL, Abcam, AB193555) and a MAB to human cytokeratin 7 (1 mg/mL, Abcam, AB9021). Cell-bound primary antibody was then detected with goat anti-Rabbit IgG Alexa 594 (1 mg/mL, Life Technologies) and goat anti-mouse IgG Alexa 488 (1 mg/mL, Life Technologies). Coverslips were adhered to corning pre-cleaned single-frosted slides via Prolong Antifade Mounting Reagent with DAPI (Invitrogen).

Immortalized HESCs culture and treatments

The immortalized human endometrial stromal cell line (HESC) was generously provided by Prof. Haibin Wang (Xiamen University) and cultured according to the manufacturer's instructions (Krikun *et al.* 2004). HESCs were cultured in phenol red-free DMEM/F-12 (Gibco) containing 10% (v/v) charcoal-stripped fetal bovine serum (CS-FBS, PAN-Biotech, Bavaria, Germany), 3.1 g/L glucose (Sigma Aldrich) and 1 mM sodium pyruvate (Sigma-Aldrich) supplemented with 50 mg/mL penicillin-streptomycin (Gibco), 1.5 g/L sodium bicarbonate (Sigma-Aldrich), 500 ng/mL puromycin (Sigma-Aldrich) and 1% insulin-transferrin-selenium (Gibco).

The *in vitro* stromal cell decidualization was performed as described elsewhere (Szwarc *et al.* 2018). The endometrial stromal cells were treated with medium containing 1% CS-FBS, 10^{-8} ME (Sigma-Aldrich) plus 10^{-7} MP (medroxyprogesterone-17-acetate, Abcam) and 0.5 mM dibutyl cAMP (Sigma-Aldrich). The media were changed every 48 h, and the cultures were maintained for up to 8 days.

We cultured both types of HDSC by DSC culture media containing hormonal cocktail (10^{-8} ME plus 10^{-7} M MPA, and 0.5 mM db-cAMP) with 10% EVs-depleted FCS for 48 h and collected the conditioned medium to isolate HDSC-sEVs.

Immortalized extravillous trophoblast cell line HTR-8/SVneo and treatment

The EVT cell line HTR-8/SVneo was purchased from American Type Culture Collection. The cell line is generated from the primary culture of cytotrophoblast cells obtained from explant cultures of human first-trimester placenta, as described elsewhere (Graham *et al.* 1993). HTR-8/SVneo cells were cultured in RPMI 1640 (Gibco) supplemented with 10% FBS (PAN-Biotech) and 50 mg/mL penicillin-streptomycin.

For the experiments described, cells were seeded in six-well tissue culture plates until 60–80% confluence, cultured in

FBS-free media for 12–14 h and then treated with HDSC-sEVs (25 µg/mL, 50 µg/mL) for 48 h.

Isolation of HDSC-sEVs

HDSC-sEVs were isolated from the cell culture supernatant as described elsewhere (Wu *et al.* 2018) with some modifications. In brief, the culture supernatant of human stromal cells was centrifuged at 300g for 10 min, 2200g for 30 min at 4°C, and 10,000g for 45 min at 4°C to eliminate cells and debris. The supernatant fluid was filtered using a 0.22 µm sterile filter (Millipore, Billerica) and then ultracentrifuged at 100,000g for 70 min at 4°C. The pellets were resuspended in a larger volume of PBS and ultracentrifuged at the same high speed for an additional 70 min.

Identification of HDSC-sEVs

For transmission electron microscopy analysis, sEVs were fixed in 2% glutaraldehyde, planted onto carbon-coated grids and stained with phosphotungstic acid. Grids were examined using a transmission electron microscope (H-7650, Hitachi High-Technologies, Tokyo, Japan). We tested sEVs classical markers such as Alix (1:1000, CST), CD81 (1:1000, Abcam), CD63 (1:1000, Abcam) and GAPDH (1:1000, CST). Western blotting was performed as previously described (Li *et al.* 2014).

Fluorescence labeling of HDSC-sEVs

HDSC-sEVs purified from cell cultured media were resuspended in diluent C from the lipophilic PKH-67 labeling kit (Sigma-Aldrich) and labeled according to the manufacturer's instructions. In brief, HDSC-sEVs were incubated in diluent C at 37°C for 10 min. Excess dye was removed by washing with PBS and ultracentrifugation at 100,000g for 70 min at 4°C. The labeled sEVs were pelleted by PBS and stored at –80°C (maximum of 4 weeks) for downstream experiments, including uptake assays.

Uptake of HDSC-sEVs by trophoblast cells

HTR8/SVneo cells and primary human EVT cells were plated on chamber slides, treated with a final concentration of 28 ng/mL labeled HDSC-sEVs (Green) for 4 h, fixed with 4% paraformaldehyde at room temperature for 20 min, permeabilized with ice-cold acetone at room temperature for 5 min and subsequently stained with DAPI (blue) as per the manufacturer's recommendations (Pantra, Guangzhou, China). Confocal images of trophoblast cells were acquired on a Zeiss Pascal system (Zeiss LSM 880). Z-stack images of HTR8/SVneo cells were recorded using a z-step size of 0.5 µm and acquired on a Zeiss Pascal system. Image processing was performed using the ZEN 2 software (Zeiss), and 3D data sets were deconvolved using SoftWoRx (Applied Precision, GE Healthcare). For the analysis of cellular internalization of HDSC-sEVs, images were also deconvolved using ZEN 2 software.

Matrigel invasion assay of HTR8/SVneo cells treated with HDSC-sEVs

The upper chamber of 8 μ m pore-sized Transwell inserts (Corning) was pre-coated with 60 μ L of pre-diluted Matrigel (Corning). The 2×10^4 pre-starved HTR8/SVneo cells were suspended in 250 μ L RPMI-1640 (Gibco) with HDSC-sEVs (25 μ g/mL, 50 μ g/mL) and 0.1% EVs-depleted FCS. Cell suspensions were seeded in upper chambers for 48 h. RPMI-1640 supplemented with 10% EVs-depleted FCS was added to the lower chamber as a chemoattractant. After incubation for 48 h, the cells in the upper side of the insert were removed with a cotton swab and the cells on the lower side were fixed in absolute methanol for 20 min and stained with 0.1% crystal violet (Beyotime, Shanghai, China) for 30 min. Five microscopic fields on the membrane of the chamber were examined under an Olympus IX 71 inverted microscope (Olympus) for counting the number of crystal violet-positive cells, which represent the invasive cells. Mean values from a minimum of five experiments were used in the analysis.

Quantitative real-time polymerase chain reaction analysis

Total RNA extracted from HTR8/SVneo cells were treated with HDSC-sEVs (25 μ g/mL, 50 μ g/mL), and primary trophoblasts were treated with primary HDSC-sEVs (25 μ g/mL, 50 μ g/mL) for 24 h and processed for RNA extraction. The transcript levels of N-cadherin were assessed by real-time PCR, as described elsewhere (Li *et al.* 2014). The primers used for real-time PCR were N-cadherin, 5'-GGA CAG TTC CTG AGG GAT CA-3' (forward) and 5'-GGA TTG CCT TCC ATG TCT GT-3' (reverse); human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-GAG TCA ACG GAT TTG GTC GT-3' (forward) and 5'-GAC AAG CTT CCC GTT CTC AG-3' (reverse); SMAD2, 5'-GCC TTT ACA GCT TCT CTG AAC AA-3' (forward) and 5'-ATG TGG CAA TCC TTT TCG AT-3' (reverse); SMAD3, 5'-CCC CAG CAC ATA ATA ACT TGG-3' (forward), and 5'-AGG AGA TGG AGC ACC AGA AG-3' (reverse). Expression levels were determined using the $2^{-\Delta\Delta C_t}$ threshold cycle method. All experiments were performed five times.

Western blotting

Western blotting was performed as previously described (Li *et al.* 2014). The cells were washed three times in cold PBS and lysed in ice-cold lysis buffer (KeyGen Biotech, Nanjing, China). Total protein concentration was determined by BCA method (Thermo Fisher). Protein samples were loaded onto 10% (v/v) SDS-PAGE, resolved at 120 V for 70 min, transferred to nitrocellulose membranes (Millipore) and subsequently blocked with 5% BSA (Sigma-Aldrich). After blocking, the membranes were incubated overnight at 4°C with primary antibodies against N-cadherin (1:1000, CST, MA, USA), (1:1000, CST), Phospho-SMAD2 (1:1000, CST), Phospho-SMAD3 (1:1000, CST), SMAD2 (1:1000, CST), SMAD3 (1:1000, CST), and GAPDH (1:1000, CST). The blots were washed and incubated with a secondary antibody conjugated with peroxidase (goat anti-Rabbit IgG, Santa

Cruz). Immunolabeling was performed using the ECL Western Blotting Substrate Pierce Kit (Thermo Scientific) in the Tanon 6200 Luminescent Imaging Workstation (Tanon, Shanghai, China). GAPDH was used as the loading control.

Small interfering RNA transfection

siRNAs for targeting N-Cadherin, SMAD2, SMAD3, and scrambled negative control siRNA were purchased from Guangzhou RiboBio Co. Ltd. Approximately 50% confluent cells were transferred with 20 nM siRNA and 20 nM of its negative control by lipofectamine 2000 (Invitrogen), in accordance with the manufacturer's instructions. RT-qPCR and Western blotting analysis were performed to examine the transfection efficiency. Twenty-four hours post-transfection, the HTR-8/SVneo cells were treated with HDSC-sEVs (25 μ g/mL).

Statistical analysis

All values were shown as means $\pm$ SEM of at least five independent experiments. Nonparametric test was performed. Statistical evaluation of the data was performed by Kruskal–Wallis test for comparisons among multiple groups and by the Mann–Whitney test for comparisons between two groups as appropriate. Statistical analysis was performed using SPSS19.0 program. The difference was regarded statistically significant if the two-tailed *P*-value was <0.05 .

Results

Isolation and identification of HDSC-sEVs in culture media

We isolated the HDSC-sEVs from the culture medium of immortal human decidual stromal cells. The isolated sEVs were identified by electron microscopy and Western blotting. The electron microscopic image of typical sEVs is demonstrated in Fig. 1A. Meanwhile, the isolated sEVs were identified by the presence of endosomal markers, CD63, tetraspanin CD81, and Alix (Fig. 1B). We also isolated HDSC-sEVs from the culture media of primary human decidual stromal cells. The purity of isolated human DSCs was assessed by positive immunocytochemical staining of the fibroblast marker, vimentin, and negative expression of the trophoblast marker, CK7 (Supplementary Fig. 1A, see section on [supplementary materials](#) given at the end of this article). Immunocytochemistry has shown that vimentin-positive cells made up for above 95%. Primary HDSC-sEVs were identified by electron microscopy and Western blotting (Supplementary Fig. 1B and C).

Uptake of HDSC-sEVs by trophoblast

We hypothesized that HDSC-sEVs modulate the uterine microenvironment via a paracrine mechanism after their uptake by the adjoining cells. To determine the uptake of HDSC-sEVs by placental trophoblast cells, we investigated HDSC-sEVs uptake kinetics in HTR-8/

Figure 1 Identification of human decidual stromal cells-derived small extracellular vesicles (HDSC-sEVs) and its uptake by trophoblast cells. (A) Morphology of HDSC-sEVs was confirmed by transmission electron microscopy. Scale bar, 200 nm. (B) Positive markers (Alix, CD63 and CD81) of HDSC-sEVs were detected by Western blotting. (C) Human extravillous trophoblast cells (EVTs), HTR8/SVneo, were pre-treated with PKH67-labeled HDSC-sEVs for 4 h followed by DAPI (blue) staining for confocal microscopy analysis. Scale bar, 20 μ m. (D) Representative 3D images (xy/yz/xz) show co-localization of HDSC-sEVs (green) within HTR8 cells. Scale bar, 5 μ m.

SVneo cells (human first-trimester cytotrophoblast cell line usually used in trophoblast biology research) and primary human EVTs. After co-incubation of PKH-67-labeled HDSC-sEVs with HTR8/SVneo cells for 4 h, the cells were washed and fixed. Subsequently, the internalization of labeled HDSC-sEVs by HTR-8/SVneo cells was examined using confocal fluorescence microscopy. Confocal microscopy and z-zack images revealed the uptake of PKH-67-labeled HDSC-

sEVs by HTR-8/SVneo cells (Fig. 1C and D). We also investigated primary HDSC-sEVs uptake kinetics in primary human EVTs where the purity was assessed by positive immunocytochemical staining of trophoblast marker CK7 (Supplementary Fig. 1D), and results show that primary human EVTs can uptake PKH-67-labeled primary-HDSC-sEVs (Supplementary Fig. 1E). These results suggested that the HDSC-sEVs may be involved in the regulation of trophoblast cells function.

HDSC-sEVs induce trophoblast invasion

To investigate whether HDSC-sEVs affect trophoblast invasion, we used transwell matrigel invasion assay, including matrigel-precoated polycarbonate transwell filters with HTR-8/SVneo cells. The results showed that HDSC-sEVs induced significant HTR8/SVneo cell migration to the undersurface of the transwell filters compared with that without HDSC-sEVs (Fig. 2A and B). HDSC-sEVs significantly increased the invasive ability of HTR-8/SVneo cells at concentrations of ≥ 25 μ g/mL.

Exposure to HDSC-sEVs increases N-cadherin gene expression and protein levels in trophoblast cells

To determine the mechanism by which HDSC-sEVs increase the invasiveness of trophoblast cells, we measured the expressions of N-cadherin in the HTR-8/SVneo cells. EMT is a well-recognized process whereby epithelial cells differentiate into a mesenchymal phenotype (Kalluri 2009). N-cadherin is one of the typical markers of EMT and plays a key role in trophoblast invasion (Zhao *et al.* 2018). Therefore, we assessed the mRNA expression of N-cadherin in trophoblasts by RT-qPCR. We found that the mRNA expression of N-cadherin was significantly increased in HTR-8/SVneo cells treated with HDSC-sEVs (Fig. 2C) or primary EVT cells stimulated by primary HDSC-sEVs after 24 h (Fig. 2D). Further, Western blotting analysis revealed a significant increase in the protein expression of N-cadherin in HDSC-sEVs-treated HTR8/SVneo cells or primary HDSC-sEVs-treated primary EVT cells after 48 h stimulation (Fig. 2E and F).

N-cadherin is involved in HDSC-sEVs-induced trophoblast invasion

To determine whether N-cadherin increase was involved in HDSC-sEVs-induced HTR8/SVneo cells invasion, we used siRNA-mediated knockdown of N-cadherin. Transfection of HTR8/SVneo cells with siRNA targeting N-cadherin for 24 h suppressed both basal and HDSC-sEVs-induced N-cadherin protein levels (Fig. 3A, B and C). Matrigel-coated transwell invasion assays showed that the decrease of N-cadherin impaired the invasion of both basal and HDSC-sEVs-induced HTR8/SVneo cells (Fig. 3D and E).

Figure 2 HDSC-sEVs significantly increased the invasiveness of trophoblast cells and induced N-cadherin expression. (A and B) Matrigel-coated transwell assay reveals the invasiveness of HTR8/SVneo with or without HDSC-sEVs (25 µg/mL and 50 µg/mL) pre-treatment. The numbers of invasive cells were quantified in five randomly selected visual fields, and a summary of the statistical analysis was showed. Magnification 100×, scale bar, 100 µm. (C and D) HTR8/SVneo cells were treated with or without HDSC-sEVs (25 µg/mL and 50 µg/mL) for 24 h (C) or primary EVT cells were treated with or without primary HDSC-sEVs (25 µg/mL and 50 µg/mL) for 24 h (D) and N-cadherin expression level was evaluated by RT-qPCR with GAPDH as the internal control. (E) Protein level of N-cadherin was detected by Western blotting in HTR8/SVneo cells with or without HDSC-sEVs pre-treatment (25 µg/mL and 50 µg/mL) for 48 h. (F) Protein level of N-cadherin was detected by Western blotting in primary EVT cells with or without primary HDSC-sEVs pre-treatment (25 µg/mL and 50 µg/mL) for 48 h. Data were normalized to GAPDH. The results are represented as the means ± SEM. $n = 5$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

SMAD2/3 signaling is involved in HDSC-sEVs-induced elevation of N-cadherin

To determine whether HDSC-sEVs induces HTR8/SVneo cells invasion in a SMAD-dependent manner, we tested SMAD2 and SMAD3 phosphorylation by Western

blotting after the stimulation of HTR8/SVneo cells with 25 µg/mL HDSC-sEVs for 30 or 60 min. HDSC-sEVs significantly increased phosphorylation of SMAD2 and SMAD3 (Fig. 4A). Then we used siRNA to specifically knock down total and phosphorylated SMAD2/3, and the related effects were verified by RT-qPCR and Western blotting (Fig. 4B, C and D). To investigate the combined role of SMAD2 and SMAD3 in HDSC-sEVs-induced increase in N-cadherin, HTR8/SVneo cells were treated with 25 µg/mL HDSC-sEVs after siRNA-mediated depletion of SMAD2 and SMAD3 and the induction of N-cadherin treated with HDSC-sEVs in HTR8/SVneo cells was inhibited by transfected si-Smad2+si-Smad3. The results showed that knockdown of SMAD2 and SMAD3 significantly abolished the stimulatory effects of HDSC-sEVs on N-cadherin mRNA and protein expressions (Fig. 4E and F).

Discussion

Here, we demonstrated that human decidual stromal cells could release HDSC-sEVs that induce human trophoblast invasion. In the *in vitro* study, we induced decidualization of immortal human endometrial stromal cells, isolated primary human decidual stromal cells and collected HDSC-sEVs and primary HDSC-sEVs from the culture media. Then, we identified the HDSC-sEVs by morphological analysis and endosomal markers. We demonstrated the uptake of HDSC-sEVs by trophoblast cells *in vitro* and found that HDSC-sEVs could significantly stimulate the invasive potential of trophoblast cells. Our data indicated a significant increase in N-cadherin expression in trophoblast cells stimulated by HDSC-sEVs, and knockdown of N-cadherin by RNAi attenuated this effect. Besides, we observed the upregulation of phosphorylation of SMAD2/3 after HDSC-sEVs treatment. Our findings suggest that HDSC-sEVs promoted the invasion of trophoblast cells via the upregulation of N-cadherin through SMAD2/3 signaling pathway. The present study suggested that human decidual stromal cells may participate in trophoblast invasion during embryo implantation and placentation by releasing HDSC-sEVs.

Trophoblast invasion plays an essential role in successful implantation and placentation. In contrast to uncontrolled tumor invasion, the invasion of trophoblast cells is coordinated and controlled by numerous cytokines. Incomplete invasion of trophoblast cells and impaired reconstruction of spiral arteries in decidua result in pregnancy disorders such as PE and fetal growth restriction (Fitzgerald *et al.* 2008). During embryo implantation and invasion, trophoblasts invade the decidual tissues, which bring them in close contact with the decidual stromal cells. Besides, decidual stromal cells can release many molecules that modulate the trophoblast cells invasion (Sharma *et al.* 2016). However, the extensive cross-talk between decidual

Figure 3 HTR8/SVneo cells invasion was attenuated by siRNA mediated N-cadherin knockdown. (A) HTR8/SVneo cells were transfected with 20 nM nontargeting control siRNA (si-control) or siRNA targeting N-cadherin (si-N-cadherin) for 24 h before HDSC-sEVs treatment. After 24 h treatment with or without HDSC-sEVs, mRNA level of N-cadherin was detected by RT-qPCR using GAPDH as the internal control. (B and C) After 48 h treatment with or without HDSC-sEVs, protein levels of N-cadherin in HTR8/SVneo cells were detected by Western blotting, and data were normalized to GAPDH. (D and E) The effects of N-cadherin knockdown on HDSC-sEVs-induced invasion of HTR8/SVneo cells were determined by transwell assay. Representative images were shown. Magnification 100 $\times$, scale bar, 100 μ m. The results are represented as the means $\pm$ SEM. $n=5$. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

stromal cells and trophoblast cells, especially the mechanism by which decidual stromal cells regulate trophoblast invasion, is not completely understood. Our study suggested human decidual stromal cells may regulate trophoblast invasion by releasing sEVs.

EVs are vesicles released by most cells including exosomes, microvesicles and apoptotic body. sEVs represent a particular subtype of extracellular vesicles with the diameter of 40–300 nm, and sEVs contain various signaling molecules, including mRNA, microRNA, lncRNA and proteins (Raposo & Stoorvogel 2013, Théry *et al.* 2018). sEVs play an important role in cell-to-cell crosstalk and are involved in many physical and pathological events including pregnancy (Homer *et al.* 2017). During normal pregnancy, the plasma concentration of sEVs increases significantly compared with non-pregnant women (Sabapatha *et al.* 2006). sEVs isolated from pregnant women can reportedly promote endothelial cell migration, indicating that sEVs in plasma of normal pregnant women may have bioactive functions (Salomon *et al.* 2014). Besides, the total concentration of sEVs in preeclamptic women was reported to be significantly higher than that of normal pregnant women, indicating that sEVs may contribute to the occurrence of PE (Pillay *et al.* 2017). In contrast to many studies focused on sEVs derived from syncytiotrophoblast cells, few studies investigated the role of HDSC-sEVs during embryo implantation and placentation. In this study, we collected HDSC-sEVs and performed transwell

chamber assay to investigate the effect of HDSC-sEVs on invasion capacity of trophoblast cell line HTR8/SVneo cells. It turned out that HDSC-sEVs could significantly promote the invasiveness of HTR8/SVneo cells, which suggested that HDSC-sEVs may play an important role in trophoblast cells invasion. We further investigated the mechanism of HDSC-sEVs in regulating trophoblast invasion, and elevated expression level of N-cadherin, hallmark of EMT, was detected in trophoblast cells. EMT is characterized by a decrease in the expression of epithelial markers, such as E-cadherin, and increase in the expressions of mesenchymal markers, such as N-cadherin and vimentin (Christiansen & Rajasekaran 2006, Xu *et al.* 2009). Trophoblast invasion is closely associated with EMT, and abnormal EMT may be involved in the pathogenesis of pregnancy-related disorders (DaSilva-Arnold *et al.* 2018). N-cadherin is one of the typical markers of EMT, and many studies pointed out that N-cadherin played a key role in trophoblast invasion (Li *et al.* 2014, Zhao *et al.* 2018). Our study focused on the role of N-cadherin in HDSC-sEVs promoting trophoblast invasion, and we found that HDSC-sEVs induced N-cadherin expression and invasiveness of trophoblast cells could be blocked when N-cadherin was knocked down. In our subsequent experiments, we found that HDSC-sEVs could upregulate N-cadherin expression through SMAD2/3 signaling. SMAD2/3 are important transcription factors in TGF- β superfamily signaling pathway. This signaling pathway is involved

Figure 4 SMAD2/3 signaling mediates the HDSC-EVs-induced upregulation of N-cadherin. (A) HTR8/SVneo cells were treated with or without 25 $\mu\text{g}/\text{mL}$ HDSC-sEVs for different lengths of time (30 or 60 min). Phosphorylated SMAD2 and SMAD3 (p-SMAD2 and p-SMAD3) levels were detected by Western blotting with corresponding phospho-specific antibodies. (B) HTR8/SVneo cells were transfected with 20 nM scrambled negative control siRNA (si-control) or siRNA targeting SMAD2 + SMAD3 (si-SMAD2 + 3) for 24 h, and mRNA levels of SMAD2/SMAD3 were tested by RT-qPCR using GAPDH as reference. (C) SMAD2 protein and p-SMAD2 protein expressions were significantly reduced in HTR8/SVneo cells transfected with si-SMAD2 + 3, compared to scramble transfected cells. (D) SMAD3 protein and p-SMAD3 protein expression were significantly reduced in HTR8/SVneo cells transfected with si-SMAD2 + 3, compared to scramble transfected cells. (E) After being transfected with si-SMAD2 + 3 or si-control for 24 h, HTR8/SVneo cells were treated with or without 25 $\mu\text{g}/\text{mL}$ HDSC-sEVs. After 24 h, N-cadherin mRNA levels were detected by RT-qPCR using GAPDH as reference. (F) After 48 h, protein levels of N-cadherin in HTR8/SVneo cells were detected by Western blotting and the data were normalized to GAPDH (E and F). The results are represented as the means $\pm$ SEM. $n = 5$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

in the modulation of hormone production and cancer progression (Holtzhausen *et al.* 2014).

The sEVs contents are diverse and are regulated by the microenvironment (Homer *et al.* 2017). Menstrual cycle hormones can modulate the cargoes of endometrial epithelial-derived sEVs. Previous study demonstrates that the molecular contents are different between estrogen-treated endometrial epithelial-derived exosomes and estrogen plus progesterone-treated endometrial epithelial-derived exosomes, and many proteins are not reported before in exosomes database (Greening *et al.* 2016). During embryo implantation and placentation, significant changes occur within the uterine microenvironment, such as changes in hormone levels, and it will be interesting to further explore the unique molecules in HDSC-sEVs cargo compared with related database (Greening *et al.* 2016). Proteomic analysis should be performed to identify the specific molecules of HDSC-sEVs that regulate the SMAD2/3-N-cadherin

pathway to modulate the invasiveness of trophoblast cells. Besides, more experiments should be performed on primary human trophoblast to further identify the role of HDSC-sEVs on trophoblast bioactivities during implantation and placentation.

In summary, our study demonstrated that the HDSC-sEVs could induce the invasion of trophoblast cells through the SMAD2/3-N-cadherin signaling pathway. Decidua could modulate the function of trophoblast cells by releasing HDSC-sEVs. Our study provides new insights into the interaction of trophoblasts and decidual stromal cells. It may help us to further understand the mechanisms of the occurrence of pregnancy-related disorders. Further studies should be performed to investigate the diverse changes in HDSC-sEVs under different uterine milieu and their involvement in many pregnancy-related disorders. The particular contents of HDSC-sEVs in different uterine microenvironments should be revealed to demonstrate

the signaling pathways that regulate the bioactivities of trophoblast cells.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-18-0616>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

M L and X C conceived the ideas and designed the experiments. Y X W, X J Y and F S performed the experiments. Q X C, L P H and Y X L provided the patient samples for scientific investigation. S J J H Y H analyzed the data. M L, R H wrote the paper. S Q, Y H Y led the project, interpreted the data and drafted critical revisions.

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