Silencing SEC5 inhibits trophoblast invasion via integrin/Ca²⁺ signaling

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Abstract

The invasion of maternal decidua by extravillous trophoblast (EVT) is essential for the establishment and maintenance of pregnancy, and abnormal trophoblast invasion could lead to placenta-associated pathologies including early pregnancy loss and preeclampsia. SEC5, a component of the exocyst complex, plays important roles in cell survival and migration, but its role in early pregnancy has not been reported. Thus, the present study was performed to explore the functions of SEC5 in trophoblast cells. The results showed that SEC5 expression in human placental villi at first trimester was significantly higher than it was at the third trimester, and it was abundantly localized in the cytotrophoblast (CTB) and the trophoblastic column. SEC5 knockdown was accompanied by reduced migration and invasion in HTR-8/SVneo cells. In addition, the expression and plasma membrane distribution of integrin β 1 was also decreased. Furthermore, shRNA-mediated knockdown of SEC5 inhibited the outgrowth of first trimester placental explants. SEC5 and InsP₃R were colocalized in the cytoplasm of HTR-8/SVneo cells, and the cell-permeant calcium chelator BAPTA-AM could significantly inhibit HTR-8/SVneo cell invasion. The Ca²⁺ imaging results showed that the 10% fetal bovine serum-stimulated cytosolic calcium concentration ([Ca²⁺]_c) was not only reduced by downregulated SEC5 but also was blocked by the InsP₃R inhibitor. Furthermore, either the [Ca²⁺]_c was buffered by BAPTA-AM or the knockdown of SEC5 disrupted HTR-8/SVneo cell F-actin stress fibers and caused cytoskeleton derangement. Taken together, our results suggest that SEC5 might be involved in regulating trophoblast cell migration and invasion through the integrin/Ca²⁺ signal pathway to induce cytoskeletal rearrangement.

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Introduction

During early pregnancy, cytotrophoblasts (CTBs) differentiate into syncytiotrophoblasts (STBs) and extravillous cytotrophoblasts (EVTs) (Pollheimer *et al.* 2018). Invasive EVT migrate into the maternal uterus and anchor the placenta to the uterine wall to remodel maternal spiral arteries for nutrient transport to the developing fetus. Interference with these processes would cause different placenta-associated pathologies. Insufficient trophoblast invasion is closely related to recurrent miscarriage (RM), preeclampsia (PE) and intrauterine growth restriction (IUGR) (Reister *et al.* 2001, Staun-Ram & Shalev 2005), while excessive trophoblast invasion leads to placenta accreta and percreta (Tantbirojn *et al.* 2008). However, although the invasion of EVT has been studied comprehensively, its underlying molecular mechanisms are still unknown.

Trophoblast proliferation, migration and invasion are regulated by complex signaling pathways, which

involve a variety of factors at the maternal-fetal interface, including growth factors, hormones and the extracellular matrix (ECM) (Bischof *et al.* 2000, Cakmak & Taylor 2011). Integrins, a type of glycoprotein composed of different α and β subunits are a family of cell-surface receptors that integrate membrane complexes, linking the ECM to the actin cytoskeleton (De Franceschi *et al.* 2015). Integrins are important regulators of trophoblast invasion through FAK-dependent or -independent signaling pathways (Irving & Lala 1995, Arimoto-Ishida *et al.* 2009, Zhang *et al.* 2018). SEC5, also known as EXOC2, is one of the exocyst components, and modulates many physiological processes not just as a subunit, but also independent of exocyst complex (Tanaka *et al.* 2017). The protein structure of SEC5 is highly conserved, and its homology in human and mouse proteins is up to 94%. SEC5 plays important roles in vesicle trafficking, cell survival and cell motility (Issaq *et al.* 2010, Pathak *et al.* 2012, Tan *et al.* 2015), and it was found to be

expressed in the human placenta (Gonzalez *et al.* 2014). However, to the best of our knowledge, the role of SEC5 in early pregnancy has not been reported. Previously, we determined the miRNA expression profiles in human placenta villi collected from RM patients and women with normal pregnancies by deep sequencing, and we found that miR-3074-5p expression was significantly higher in RM patients (Gu *et al.* 2015). Furthermore, we found that in human trophoblast cell line HTR-8/SVneo, miR-3074-5p promoted cell apoptosis but inhibited cell invasion (Gu *et al.* 2018). Given that SEC5 is a predicted target gene of miR-3074-5p, we hypothesized that SEC5 might also be involved in the regulation of trophoblast cell invasive activity.

Thus, in the present study, the expression and distribution of SEC5 in mouse pre-implantation embryos, the implantation site during early pregnancy, and human placental tissues were determined. Additionally, by using HTR-8/SVneo as the EVT cell model and placenta villous explants as a source of EVTs, the *in vitro* effects of downregulated SEC5 expression on EVT cell migration and invasion activities as well as on the expression and plasma membrane distribution of integrin $\beta 1$, the influences on intracellular calcium signaling and trophoblast cytoskeleton rearrangement were observed to explore the roles of SEC5 in regulating EVT cell invasion activity.

Materials and methods

HTR-8/SVneo cells culture and treatment

HTR-8/SVneo cells, the immortalized human trophoblast cell line derived from chorionic villi explants of human first-trimester placenta were kindly provided by Dr Hongmei Wang, Institute of Zoology, Chinese Academy of Sciences, China. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% Antibiotic-Antimycotic (100 $\times$) (Invitrogen, Thermo Fisher Scientific) at 37°C in a humidified atmosphere of 5% CO₂. The siRNA against SEC5 (SEC5 siRNA: 5'-GGUCGGAAGACAAGGCAGdTdT-3', and the negative control siRNA (NC: 5'-UUCUCCGAACGUGUCACGUTT-3') were synthesized by Shanghai GenePharma Co., Ltd. Transfections were performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Thermo Fisher Scientific). To create polyclonal cell lines expressing the SEC5-specific shRNA and negative control stably, the lentivirus constructed and packaged by Shanghai Genechem Co., Ltd. was used to infect HTR-8/SVneo cells. The cells expression HU6-MCS-Ubiquitin-EGFP-IRES-puromycin vector or expression recombinant SEC5 shRNA were grown in the medium supplemented with puromycin at 1 μ g/mL for approximately 14 days to eliminate the cells uninfected. Western blotting assay was employed to determine whether the infected cells expression EGFP were knocked down by shRNA of SEC5 compared to negative control cells. Then, the selected cells were continued to be grown in medium supplemented with puromycin at 0.5 μ g/mL.

Embryo harvest, in vitro culture and immunofluorescent staining

Superovulation was induced by administering 7.5 IU PMSG followed 48 h later by 7.5 IU HCG to C57BL6 $\times$ DB2 F1 6-8-week-old female mice ($n=10$) obtained from the SIPPR/BK Laboratory Animal Company (Shanghai, China). Experiments were conducted in full compliance with standard laboratory animal care protocols that were approved by the Institutional Animal Care Committee of Shanghai Institute of Planned Parenthood Research (#2018-14). For embryo collection, zygotes were produced by *in vitro* fertilization (IVF) procedures. Superovulated females were killed 15–17 h post hCG injection for oocyte collection, while males were killed 1 h prior to IVF for sperm collection and capacitation. Sperm was excised from the epididymis into 1 mL HTF medium (Milipore), placed in the incubator at 37°C, with a 5% CO₂ atmosphere for 45 min. Oocyte-cumulus complexes intended for IVF were released from the oviduct into pre-warmed (at 37°C) M2 medium (Milipore) placed directly into fertilization dishes in 100 μ L micro droplets of HTF medium for 6 h under mineral oil 37°C in a 5% CO₂ atmosphere and inseminated with capacitated sperm. Oocytes were then washed with KSOM medium (Milipore) and were examined for the presence of two polar bodies and two pro-nuclei. The fertilized oocytes were moved into 20 μ L micro drops of KSOM medium under mineral oil equilibrated. All oocyte and embryo manipulations were carried out on a heated microscope stage that was maintained at 37°C. Embryos were fixed in 4% paraformaldehyde (PFA) for 30 min, permeabilized using 0.2% Triton X-100 and 0.3% Tween 20 (Sigma-Aldrich Inc.) for 15 min, washed in PBS for three times, and blocked in 10% donkey serum and 2% BSA for 1 h at room temperature. Embryos were then incubated with anti-SEC5 antibody and Cy3-conjugated secondary antibody (the information of antibodies is in Supplementary Table 1, see section on [supplementary materials](#) given at the end of this article). Next, embryos were washed four times with PBS before being mounted by mounting solution. Confocal images were acquired with a Nikon A1R confocal system.

Preparation of mouse uterus tissues from the early pregnancy phase

Adult ICR mice aged 8–10 weeks were obtained from the SIPPR/BK Laboratory Animal Company (Shanghai, China). Female mice ($n=30$) were mated with fertile males ($n=10$) to achieve pregnancy (the day a vaginal plug was observed was identified as pregnancy day 0.5). Trypan blue dye solution (0.1% in saline (w/v), 0.1 mL per mouse, Sigma) was injected via the tail vein on day 5.5 to visualize the implantation sites. The uterine tissues at the implantation sites were collected from the pregnant mice on days 5.5–8.5 of pregnancy ($n=3$, per day), fixed in 4% PFA and embedded in paraffin.

Human placental tissue collection and first trimester villous explant cultures

Frozen and formalin-fixed first trimester (6–10 weeks of gestation) and third trimester (37–40 weeks of gestation)

placental tissues were collected for Western blotting and immunofluorescence assay, respectively. The subjects were recruited from April to October of 2017 at the outpatient department of Gynecology and Obstetrics, The Second Hospital of Tianjin Medical University, China. Fresh first trimester placental villous tissues were collected for explant cultures. Human placental tissues from the first trimester were obtained from ultrasound-dated viable singleton pregnancies undergoing legal abortion for nonmedical reasons, and they were maintained in culture medium until dissection. All the experiments involving humans were approved by the Medical Ethics Committees of The Second Hospital of Tianjin Medical University and Shanghai Institute of Planned Parenthood Research (Ref # 2013-12), and we also obtained consent to publish the research data derived from these collected samples from the recruited participants. The explant culture was performed as previously reported (Baczyk *et al.* 2009). In brief, small placental villi were dissected and placed in a 24-well culture plate that had been precoated with 200 μ L of phenol red-free Matrigel (BD Biosciences, Bedford, MA, USA). Three fragments from an individual placenta were cultured per well in DMEM/F12 medium with 20% FBS, 1% antibiotic-antimycotic, 1 mM sodium pyruvate and 1% GlutaMAX supplement (Invitrogen, Thermo Fisher Scientific). Explants were allowed to attach to the Matrigel for 2 h at 37°C in a humidified 5% CO₂ atmosphere. Then, 70 μ L of medium was added; the culture was continued overnight, and it was supplemented with serum-free culture medium and infected with the shRNA of SEC5 or a negative control virus. Placental villi were anchored on Matrigel and successfully initiated outgrowth after 24 h of culture, and then they were used for the subsequent experiments. The time point at 24 h after infection was referred to as 0 h, and the explants were observed every 24 h and photographed using confocal microscopy. Explant outgrowths (the migration distance of EVT from the cell column base to the tip of the outgrowth) were measured using ImageJ software. The average outgrowth of three wells for each treatment was analyzed. The EVTs that migrated from the explant were determined by HLA-G immunostaining.

Cell invasiveness measurement by transwell assay

Cell invasion assay was performed using a BD BioCoat™ Matrigel™ Invasion Chamber (BD Biosciences) according to the manufacturer's instructions. Briefly, culture medium was added into a 24-well plate and transwell inserts were plated into the wells for 2-h rehydration at 37°C. After 24 h of transfection, HTR-8/SVneo cells were harvested with serum-free medium, 2×10^5 cells were seeded into upper compartment of the prepared inserts, and medium supplemented with 25% FBS was added to the lower compartment to induce migration. After 24-h incubation at 37°C with 5% CO₂, the cells remaining inside of the inserts were removed using a cotton swab. Membranes were then fixed with 4% paraformaldehyde, stained with 0.1% crystal violet (Sangon Biotech, Co., Ltd. Shanghai, China), washed with ddH₂O. After air-drying the samples, ten independent fields for each group were captured by a Nikon inverted microscope. Furthermore, the whole membranes containing all the stained cells were

dissolved in methanol at 4°C for 10 min and mixed, and the absorbance at a wavelength of 560 nm was measured using a UV spectrophotometer. The invaded NC and siSEC5 cells were determined by comparing the OD560 values. The experiments in each group were repeated three times under the same conditions.

Cell migration evaluation by wound-healing assay

Wound-healing assay was performed using Ibidi Wound Healing two-well culture inserts following the manufacturer's instructions. After siRNA transfection for 24 h, HTR-8/SVneo cells were adjusted to a cell concentration of 2×10^5 cells/mL, and seeded 70 μ L/well in the two-well culture-inserts placed on 35 mm dishes. Incubated the cells at 37°C for 24 h to obtain a confluent cell layer, removed the culture-insert with sterile tweezers to form a cell free gap, and filled the dishes with 1 mL of fresh medium. Observe the cell-free gap with bright-field microscopy (Nikon Instruments Inc.). Images were captured at 0 h and 30 h. To measure the area of wound closure, cell frontiers bordering the wounds were traced using ImageJ software.

Protein extraction and Western blot analysis

To prepare whole-cell extracts, cells were washed with PBS and incubated with lysis buffer at 4°C (lysis buffer: 20 mM Tris pH 7.4, 250 mM NaCl, 1.5% Triton X-100, 2 mM EDTA, 1 mM PMSF, containing protease inhibitor cocktail), and then homogenate by ultrasonic cell disruptor. For tissue protein extraction, six first trimester (6-10 weeks of gestation) and five third trimester (37-40 weeks of gestation) of frozen placental tissues were used. 50 mg of placental villi was excised, and transferred into a microcentrifuge tube. 500 μ L of RIPA buffer (Sangon Biotech Co., Ltd.) was added, followed by homogenate using a mini-homogenizer. Supernatant of cell and tissue lysate was spin at 12,400 g for 15 min at 4°C. After that, the total protein samples were extracted and quantified using the Bradford method (Bio-Rad). Approximately 50 μ g of protein for each sample was subjected to SDS-PAGE; the separated proteins were transferred to nitrocellulose membranes (Millipore). Blots were incubated with the respective primary antibodies diluted in TBST (containing 0.1% Tween 20 and 2% BSA) for 1 h at RT. Then, blots were washed and incubated with appropriate secondary antibodies and detected using an Odyssey CLx Imaging System (LI-COR, Nebraska, USA).

Calcium imaging

HTR8-SV/neo cells were seeded in 35 mm dishes and incubated with 2 μ M Fura-2 AM (Invitrogen, Thermo Fisher Scientific) in Hanks' balanced salt solution (HBSS, Sigma-Aldrich Inc.) containing 0.04% Pluronic F-127 (Sigma-Aldrich Inc.) for 30 min, in normal culture media, at 37°C and 5% CO₂. The cells were then washed and continuously perfused with HBSS containing 1.8 mM CaCl₂ and 0.8 mM MgCl₂, pH 7.4. To measure calcium imaging, the cells were perfused with HBSS containing 10% FBS to stimulate the calcium oscillation. For argurospodin B (ARB) treatment, 8 μ M ARB was added to

HBSS buffer, and the cells were perfused for 1 min, followed by stimulating of 10% FBS. Fura-2 was alternately excited at 340 and 380 nm, and the emitted fluorescence, filtered at 510 nm, was recorded using Nikon NIS-Elements software. Individual cells were chosen as different regions of interest (ROI) and the ratio (340/380) of each ROI in all time frames were obtained after background correction.

Immunofluorescence staining

HTR8-SV/neo cells were placed on coverslips (Fisher Scientific) and incubated for 24 h. The cells were fixed using 4% PFA in DPBS, followed by blocking and permeabilization with 0.1% Igepal (Sigma-Aldrich Inc.) in DPBS with 2% BSA (Amresco, OH, USA). Primary antibodies diluted in DPBS with 2% BSA (Supplementary Table 1) were applied overnight at 4°C. The cells were subsequently washed four times with DPBS before being incubated with the appropriate secondary antibodies (Invitrogen, Thermo Fisher Scientific) as diluted in 2% BSA in DPBS. The coverslips were washed four times with DPBS before being mounted on slides using mounting solution (Thermo Fisher Scientific). Confocal images were acquired with a Nikon A1R confocal system. Pearson's correlation coefficients were calculated using ImageJ software to show the overlap between Alexa Fluor 488 and Cy3 at each colocalization area. For the tissue slides, immunofluorescence staining was performed on formalin-fixed and paraffin-embedded 5 µm sections. After being deparaffinized and rehydrated, heat-induced antigen retrieval was performed by microwave in 0.1 M sodium citrate (pH 6.0), and then the slides were allowed to cool to room temperature. The endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min. The slides were blocked with 10% normal donkey serum in PBS-T (PBS+0.1% Triton X-100) for 1 h at room temperature and incubated with primary antibodies against rabbit SEC5 (4 µg/mL) or mouse HLA-G (40 µg/mL) (Abcam) diluted in 5% BSA/PBS-T overnight at 4°C. After being washed twice with PBS-T for 10 min and then PBS for 10 min, the slides were incubated with Cy3-conjugated anti-rabbit (2.5 µg/mL) or Alexa Fluor 488-conjugated anti-mouse (2 µg/mL) secondary antibody at 37°C for 30 min. Next, the slides were washed, stained with Hoechst 33342 (Invitrogen, Thermo Fisher Scientific), and mounted.

Flow cytometry detection of integrin $\beta 1$

The HTR8-SV/neo cells were collected 48 h after siRNA transfection and resuspended in culture medium and were seeded into six-well plates at a density of 5×10^5 cells/well. The cells were cultured for 24 h, digested and collected with Enzyme-Free Cell Dissociation Solution PBS-based cell dissociation buffer (Millipore), and the cell density was adjusted to 2×10^5 cells/100 µL after the cells were counted and incubated with 0.5 µL anti-integrin $\beta 1$ -PE antibody (Supplementary Table 1) on ice for 15 min. After washing with cold PBS for twice and resuspending with PBS, fluorescence intensity was then detected by flow cytometry (LSR Fortessa, BD). Each experiment was repeated three times under the same conditions.

Statistical analysis

At least three biological replicates were performed for all experiments, unless otherwise indicated. Student's *t* test was used for statistical analyses of paired observations. Differences between means were accepted as statistically significant at the 95% level ($P < 0.05$).

Results

Expression of SEC5 in mouse pre-implantation embryos and the early implantation site

The expression of SEC5 in IVF-derived pre-implantation embryos was analyzed by immunostaining. The expression and distribution of SEC5 changed dynamically over the different stages of early embryonic development. The zygotes, two-cell and four-cell-stage embryos showed low diffused staining in the cytosol and membrane (Fig. 1A, zygote, two-cell, four-cell). However, in the eight-cell stage embryo, morula and blastocyst, SEC5 began to aggregate to the membrane of the embryos, especially in the blastocyst. Furthermore, in the blastocyst, SEC5 showed significantly intense and polarized expression in the trophectoderm (TE), which give rise to the placental CTBs, STBs and EVT cells upon embryo implantation. To explore the expression pattern of SEC5 at the implantation site during early pregnancy in mice, uterus tissues were collected on days 5.5 and 8.5 and evaluated by immunofluorescence staining. The results showed high SEC5 expression at the maternal-fetal interface (Fig. 1B and C). In addition, a strong fluorescence signal was detected in trophoblast giant cells (TGCs), as defined by expression of PL1 (Fig. 1B).

SEC5 was highly expressed in the CTBs and invasive EVTs of human placental villi

We also evaluated the expression of SEC5 protein in chorionic villous tissues of pregnant women at the first and third trimesters of normal pregnancies by Western blotting and immunofluorescence staining. The Western blotting assay showed that the SEC5 protein expression level in the human placenta villi was significantly higher at the first trimester than at the third trimester (Fig. 2A and B and Supplementary Fig. 4). Immunofluorescence in the first trimester human placenta villi sections revealed that, SEC5 was abundantly localized in the CTBs and leukocyte antigen G (HLA-G)⁺ trophoblast cells differentiating toward EVTs within trophoblast cell columns and was also observed in the STBs and a subset of cells in villous stroma (Fig. 2C and Supplementary Fig. 1). In the term placenta, SEC5 was intensely expressed in the highly invasive EVT cells that invaded into the maternal decidua as defined by HLA-G (Fig. 2D).

Figure 1 Expression of SEC5 at different stages in mouse pre-implantation embryos and during embryo implantation. (A) Embryos were generated from IVF-derived zygotes and were immunostained with rabbit anti-SEC5 antibody as described in the methods (red). The nuclei were stained with Hoechst 33342 (blue). At least three embryos were stained at each developmental stage, but only the representative embryos are shown. Scale bar=20 μ m. (B and C) SEC5 expression (red) and localization in the implantation site of the mouse uterus after implantation. Mouse uteri were obtained on days 5.5 (B) and 8.5 (C), and trophoblast giant cells were stained with P11 antibody. Arrowhead: trophoblast giant cells; E: embryo; scale bar=20 μ m.

Downregulated SEC5 expression was accompanied by reduced invasion and migration activities by HTR-8/SVneo cells

Because SEC5 is highly abundant in the CTBs and anchoring villi, we hypothesized that SEC5 might play an important role during the EVT migration and invasion of the maternal decidua. To verify this hypothesis, we

performed a Matrigel transwell cell invasion and wound-healing assays. The interference of SEC5 expression by siRNA was validated by Western blotting assay (Fig. 3A and Supplementary Fig. 5). The results showed that RNAi-mediated downregulation of SEC5 in HTR-8/SVneo cells, which was accompanied with significantly decreased invasion capacities compared with the negative control

Figure 2 Expression of SEC5 in the human placenta at the first and third trimesters. (A) Western blotting of SEC5 in human placental villi from the first ($n=6$) and the third ($n=5$) trimesters. (B) The results of three independent experiments were quantified by measuring the intensity of the SEC5 protein bands relative to the β -actin controls (** $P<0.01$). (C) Immunofluorescence staining of SEC5 (red), HLA-G (green), and nuclei (blue) in normal human placental villi (8 weeks) from the first trimester. Scale bar, 50 μ m. (D) Immunofluorescence staining in the third trimester placental tissue sections using antibodies against SEC5 and HLA-G. Hoechst 33342 staining was performed to visualize the cell nuclei. Scale bar, 50 μ m. CTB, cytotrophoblast; STB, syncytiotrophoblast; TC, trophoblastic column; and EVT, extravillous trophoblast. White arrow: CTB; green arrow: STB; and yellow arrow: EVT.

cells (Fig. 3B and C). Additional wound-healing assays confirmed the conclusion that silencing SEC5 resulted in a significantly decreased migration speed and healed area compared with the nontarget siRNA controls (Fig. 3D and E).

shRNA-mediated knockdown of SEC5 interferes with EVT migration in the villous explant culture model

Placenta villous explants derived from pregnant women at 6–9 weeks of normal gestation were cultured on Matrigel-coated plates and used to assess the role of SEC5 in EVT migration. The explant growth was visualized by confocal microscope every 24 h after lentivirus infection. The migratory ability was measured

by trophoblast outgrowth on the Matrigel surface at 96 h after lentivirus treatment. As shown in the images, the EGFP-positive cells that migrated from villous explants were successfully infected by the virus (Supplementary Fig. 2). The results showed that the lentivirus-mediated shRNA knockdown of SEC5 inhibited the outgrowth and spread of human EVT cells in first trimester placental explants (Fig. 4A and B). In addition, the immunofluorescence staining analysis showed that the cells that migrated from the villi were HLA-G (a marker of EVT cells) positive (Fig. 4C).

Downregulation of SEC5 inhibits InsP_3R -mediated intracellular calcium signaling in HTR-8/SVneo cells

Previously, we reported that SEC5 promoted InsP_3R channel activity by binding to its C-terminus, it regulated the cytosolic Ca^{2+} concentrations, and it modulated phagocytosis in macrophages (Yang *et al.* 2018). To investigate the interaction of SEC5 with InsP_3R in trophoblast cells, we first tested their localization in HTR-8/SVneo cells. The immunofluorescence assay showed that SEC5 and InsP_3R colocalized in the cytoplasm region of HTR-8/SVneo cells, and the Pearson's correlation was 0.75 (Fig. 5A). Furthermore, we performed single-cell Ca^{2+} imaging to study whether the cytosolic Ca^{2+} concentrations would be changed when endogenous SEC5 was knocked down in trophoblast cells. ShRNA-mediated SEC5 knockdown and negative control stable HTR-8/SVneo cells were established and validated by western blotting (Fig. 5D and Supplementary Fig. 6). The cells were loaded with Fura-2 AM and then the intracellular calcium concentration $[\text{Ca}^{2+}]_i$ was recorded using a computer-controlled imaging system. A 10% fetal bovine serum solution was used to stimulate intracellular calcium oscillation. The results showed that the shRNA-downregulated SEC5 expression significantly reduced the 10% FBS-stimulated $[\text{Ca}^{2+}]_i$ (Fig. 5B). In addition, the percentage of calcium oscillation-responding cells induced by 10% FBS were significantly reduced by araguspoin B (ARB, a specific InsP_3R inhibitor). These results indicated that SEC5 modulates intracellular calcium signaling in HTR-8/SVneo cells.

SEC5 knockdown inhibits the invasion of trophoblast cells through integrin/ Ca^{2+} signaling and inducing F-actin rearrangement

SEC5 modulated the intracellular calcium signaling in HTR-8/SVneo cells, and its down-regulation significantly inhibited trophoblast cell invasion and migration. We performed a transwell assay to investigate whether changes in the cytosolic calcium concentration would have any impact on the invasion ability of HTR-8/SVneo cells. The results showed that 10 μ M BAPTA-AM, a highly selective cell permeant chelator for Ca^{2+} , or 4 μ M xestospongin C (XeC), a selective InsP_3R antagonist,

Figure 3 siRNA-mediated knockdown of SEC5 inhibits HTR-8/SVneo cell invasion and migration. (A) Validation of RNA interference in SEC5 by Western blotting ($n=4$; $*P<0.05$). (B) The representative images of HTR-8/SVneo cells that were transfected with a negative control (NC) and a specific siRNA of SEC5 (siSEC5) after a Matrigel invasion assay with crystal violet staining. (C) The histogram shows the statistical results of the OD values at 560 nm for the cells in each transwell assay group that were stained after invasion and dissolved in methanol ($n=3$; $*P<0.05$). (D) The wound-healing assay shows that the downregulation of SEC5 inhibits HTR-8/SVneo migration. The wounded areas of the cells between the scratch edges were observed and calculated using ImageJ software after 30 h ($n=3$; $*P<0.05$). (E) Representative images of NC and SEC5 knockdown cells at 30 h after wounding (scale bar, 100 μ m).

significantly inhibited trophoblast cell invasion to a similar extent to that of SEC5 silencing (Fig. 6A and B). The immunoblotting demonstrated that the integrin $\beta 1$ expression was decreased by SEC5 knockdown (Fig. 6E, F and Supplementary Fig. 7). In addition, the integrin $\beta 1$ distribution on the plasma membrane was also decreased. The mean fluorescence of PE-tagged integrin $\beta 1$ was decreased by 22% in siRNA-transfected groups compared with the negative control (Fig. 6C and D).

Integrins were reported to regulate trophoblast invasion through FAK-dependent or -independent pathways. To identify whether FAK phosphorylation was involved in the SEC5-mediated and integrin-activated invasion of trophoblast cells, the expression levels of FAK and p-FAK (pY397) were evaluated by Western blot in the cells. As shown in Supplementary Fig. 3, knocking down SEC5 did not have a significant effect on FAK phosphorylation. The results indicated that SEC5 regulates trophoblast cell invasion through the FAK-independent integrin signaling pathway.

Furthermore, F-actin stress fibers labeled with rabbit anti- β -actin antibody were reduced and disorganized in either SEC5 siRNA-transfected or BAPTA-AM-pretreated cells compared with those of the control cells (Fig. 6G). Incubating with 10 μ M BAPTA-AM or knocking down SEC5 expression could significantly reduce the amount of F-actin fibers in the cells, while high actin enrichment was found at the distal tips of protrusive cell extensions (Fig. 6G, arrows).

Discussion

In this study, we demonstrated that SEC5 regulated EVT cell migration and invasion in accordance with the results determined in several different cell models *in vitro*. Our results also suggested that the integrin $\beta 1$ expression and plasma membrane distribution were significantly inhibited when SEC5 expression was downregulated. Furthermore, SEC5 regulated intracellular calcium signaling through modulated InsP_3 -mediated calcium signaling and induced F-actin cytoskeleton reorganization, leading to impaired cell motility in trophoblast cells.

The blastocyst comprises a blastocyst cavity, an inner cell mass (ICM), and a trophectoderm (TE). The TE is made up of extraembryonic cells that surround the ICM, which gives rise to the placental CTB, STB and EVT cells during implantation (Marikawa & Alarcón 2009). At the blastocyst stage, the embryo hatches from the zona pellucida, exposing the trophectoderm, and it attaches to the endometrial epithelium, which initiates a complex cascade of events that lead to the development of a placenta (Aplin & Ruane 2017). Since the embryo and endometrium interaction depends on the temporal and spatial regulation of cell-cell adhesion and invasion, in the current study, we observed SEC5 expression and distribution in zygotes and embryos at different stages, including the blastocyst. The plasma membrane aggregation of SEC5 was found in the mouse eight-cell stage embryo, morula and blastocyst.

Figure 4 Knockdown of SEC5 inhibits the outgrowth of first trimester placental explants. (A) Representative images of explants from a 6–9-week placenta. The explants were photographed at 24 h, 48 h, and 96 h after lentivirus treatment. (B) The graph shows the relative EVT outgrowth 96 h after treatment as measured and calculated with ImageJ software ($n=5$; $*P<0.05$). (C) Confocal images of immunofluorescence staining using HLA-G antibody (green) showing the EVTs that migrated from the placental explants. Scale bars=100 µm.

Interestingly, a much more intense and especially aggregated expression of SEC5 in the TE was shown in blastocyst-stage embryos. To explore the involvement of SEC5 during implantation, mouse implantation sites from the uteri were collected and evaluated via immunofluorescence staining. High SEC5 expression was detected on the maternal-fetal interface during early pregnancy, including on the TGCs, which are inherently

invasive and phagocytic and first mediate the invasion of the implanted conceptus into the maternal decidua (Simmons *et al.* 2007).

Furthermore, we analyzed the expression of SEC5 in the placental villi of women with normal pregnancies, and we found that SEC5 was highly expressed in the first-trimester placental villi compared with that of the third trimester. The immunofluorescence results showed

Figure 5 shRNA-mediated knockdown of SEC5 inhibited InsP_3R -mediated intracellular calcium signaling in trophoblast cells. (A) Confocal images showing SEC5 and InsP_3R colocalization in cytoplasm of HTR-8/SV neo cells. (a, b, c, and d) A representative resting cell was immunostained with anti-SEC5 (red) and anti- InsP_3R antibodies (green). (e) The colocalization was analyzed by the colocalization finder in ImageJ software, and the colocalized regions were highlighted. (f) The Pearson's correlation was calculated with ImageJ. (B) Ca^{2+} transient imaging in HTR-8/SVneo cells as stimulated by Ca^{2+} -free Hank's balanced salt solution, followed by the addition of 10% FBS to the extracellular solution. Representative Ca^{2+} traces depicting FBS-induced Ca^{2+} oscillation. The data were summarized as the means $\pm$ S.E.M. from three experiments, with at least 100 cells analyzed. (C) The percentage of responding cells blocked by ARB. (D) Validation of shRNA interference with SEC5 by Western blotting (* $P < 0.05$).

Figure 6 SEC5 regulating HTR-8/SVneo cell motility through InsP_3R -mediated intracellular calcium signaling and integrin/ Ca^{2+} /cytoskeleton rearrangement. (A) The trophoblast cell invasion was assessed by Matrigel invasion assay and crystal violet staining. The cells were pretreated with the indicated concentrations of BAPTA-AM and XeC for 24 h, followed by transwell assay. (B) Statistical results of the OD values at 560 nm for the cells in each group that were stained after invasion and dissolved in methanol ($n=3$; $*P<0.05$). (C and D) Plasma membrane distribution of integrin $\beta 1$ on HTR-8/SVneo cells as measured by flow cytometry. The cells were stained with PE-integrin $\beta 1$ antibody, and the mean intensity was calculated ($n=3 \times 3$; $**P<0.01$). (E and F) Representative Western blots and quantitative densitometry for integrin $\beta 1$ ($n=3$; $*P<0.05$). (G) The disposition of the F-actin cytoskeleton in the cells. The cells were probed with β -actin antibody and followed by Cy3-donkey anti rabbit secondary antibody. Representatives of at least three experiments are shown. Scale bars=20 μm .

that SEC5 was intensely localized in the CTB cells and EVTs in anchoring villi during the first trimester. As trophoblast progenitor cells, the CTB cells are the so-called epithelial 'stem cells' of the placenta to form STB and EVT cells (Chang *et al.* 2018). In anchoring villi, CTBs that reside in the villus tips differentiate into EVTs that migrate out from the placenta and invade the decidua to anchor the placenta, and they penetrate the intima of the uterine spiral arteries to establish fetomaternal circulation. Trophoblast cells display a highly proliferative and invasive capacity in first trimester placentas, during which SEC5 was also highly expressed. In addition, SEC5 reportedly played an important role in the polarized delivery of adhesion molecules and cell migration not only as a component, but it also had a function that was separate from the exocyst protein complex (Wang *et al.* 2015, Tanaka *et al.* 2017). Thus, we hypothesized that SEC5 might be involved in regulating the trophoblast cell invasion capability. As expected, we found that the knockdown of SEC5 expression through its specific siRNA or shRNA significantly inhibited the trophoblast invasion and EVT outgrowth of placenta villous explants.

It is known that during early implantation, changes in the integrin family proteins have vital functional consequences for the invasiveness of the trophoblasts (Gleeson *et al.* 2001, Na *et al.* 2012, Chung *et al.* 2016). The $\alpha 5 \beta 1$ and $\alpha 1 \beta 1$ integrins are upregulated in differentiating and invasive CTBs (Damsky *et al.* 1994). It was reported that there was a direct interaction between SEC5 and paxillin, a molecular scaffold that organizes signaling proteins that are responsible for remodeling the plasma membrane and the actin cytoskeleton to orchestrate the protrusive activity required for cell motility (Turner *et al.* 2001). Interference with exocyst activity impairs integrin delivery to the plasma membrane and inhibits tumor cell motility and matrix invasiveness (Spiczka & Yeaman 2008). In addition, the knockdown of SEC5 blocked PIPK γ 2-enhanced $\beta 1$ -integrin recruitment to the migrating cell front (Thapa *et al.* 2012). Therefore, we evaluated the expression and distribution of $\beta 1$ integrin in the human EVT cell line HTR-8/SVneo, and we found that the expression and plasma membrane distribution of $\beta 1$ integrin were decreased in SEC5-knockdown cells. This finding indicated that SEC5 might be a regulator of trophoblast cell motility by directly changing the membrane traffic of integrin or by modulating the integrin signaling pathways.

FAK is an extensively tyrosine kinase that is phosphorylated and activated in response to cell adhesion to ECM (Arimoto-Ishida *et al.* 2009, Mythreye *et al.* 2013). The phosphorylation of FAK plays a critical role in integrin-mediated signaling transduction pathways and cell motility. However, we found that FAK phosphorylation was not affected by SEC5 knockdown in HTR-8/SVneo cells (Supplementary data 4). These results

suggested that SEC5 regulated trophoblast cell invasion via the FAK-independent integrin signaling pathway.

Ca^{2+} is a second messenger that regulates numerous cellular processes, including gene expression, proliferation, differentiation, and metastasis. The intracellular Ca^{2+} concentration is organized in complex spatial and temporal patterns by an elaborate Ca^{2+} signaling system consisting of Ca^{2+} pumps, channels, and Ca^{2+} -binding proteins. The endoplasmic reticulum (ER) is the major Ca^{2+} storage organelle in cells, and the 1,4,5-trisphosphate receptor (InsP $_3$ R) is an intracellular Ca^{2+} release channel primarily located at the ER membrane, and it plays a key role in regulating intracellular Ca^{2+} signals (Mak & Foscett 2015). Recently, we reported that SEC5 interacted directly with InsP $_3$ R, leading to elevated cytoplasmic Ca^{2+} in macrophages (Yang *et al.* 2018). In this study, we found that SEC5 and InsP $_3$ R are colocalized in the cytoplasm of HTR-8/SVneo cells. During stimulation, the cytosolic Ca^{2+} concentration and signals are organized as a repeated Ca^{2+} pulse known as Ca^{2+} oscillations, which is essential for many biological processes such as cell migration. Therefore, intracellular calcium imaging was performed, and the results showed that when endogenous SEC5 was knocked down, the serum-stimulated intracellular Ca^{2+} oscillation was significantly decreased. In addition, ARB, a specific InsP $_3$ R inhibitor, reduced the percentage of calcium oscillation-responsive cells, but it did not affect the calcium oscillation peaks. These results indicated that the $[\text{Ca}^{2+}]_c$ was blocked by InsP $_3$ R inhibitor in HTR-8/SVneo cells.

The cell migration capability was reportedly dependent on Ca^{2+} signaling in different cell types. Moreover, our results showed that the buffering of intracellular Ca^{2+} with BAPTA-AM or XeC significantly inhibited trophoblast cell invasion. Cytoskeleton rearrangement and cell migration is dependent on the dynamics of intracellular Ca^{2+} signaling. Integrin-mediated cell adhesion and migration is associated with Ca^{2+} -sensitive proteins and the intracellular calcium concentration (Huttenlocher *et al.* 1997, Dourdin *et al.* 2001, Xiang *et al.* 2018). According to Giannone *et al.* (2002), serum-induced Ca^{2+} spikes in cells could be blocked by inhibitory antibodies against $\beta 1$ and $\beta 3$ integrin subunits, with the generation of these Ca^{2+} spikes depending on the activation of phospholipase C γ (PLC γ) and the production of InsP $_3$. These results indicated that serum-stimulated intracellular calcium spikes were dependent on integrin signaling and the activation of InsP $_3$ R. In addition, Alvarez *et al.* (2016) reported that ligated integrin triggered an ATP release through the activation of InsP $_3$ R and the uptake of Ca^{2+} to promote cell adhesion. In the present manuscript, the results showed that the downregulation of SEC5 decreased the expression and membrane distribution of integrin $\beta 1$. These results suggest that the SEC5 knockdown

inhibits the migration and invasion of trophoblast cells, which were dependent on the integrin/InsP₃/Ca²⁺ signal pathway.

Cell migration can be divided into three distinct stages, with protrusion, in which lamellipodia and filipodia are extended forward over the extracellular matrix; attachment, in which the actin cytoskeleton connects to the adhesion sites and interacts with other focal adhesion proteins; and traction, in which the cell body moves forward (Pai *et al.* 2001). The dynamic regulation of the filamentous actin (F-actin) cytoskeleton rearrangement plays a key role in cell adhesion and migration (Clarke & Spudich 1977). Integrin β 1 ligation initiates the recruitment of a cytoskeletal actin component and linkage with the forward-moving actin cytoskeleton (Felsenfeld *et al.* 1996). Therefore, the formation of an F-actin cytoskeleton was assessed by immunofluorescence staining. The data showed that the knockdown of SEC5 or the depletion of cytosolic Ca²⁺ significantly impaired the F-actin cytoskeletal fiber in the trophoblast cells.

In summary, this study provides evidence that SEC5 plays functional roles in trophoblast migration and invasion *in vitro*, and in the outgrowth of EVT in an explant culture model. To regulate these processes, SEC5 directly changes the expression and traffic of integrin β 1. However, SEC5 modulates intracellular calcium signaling through its interaction with InsP₃R. The disruption of intracellular calcium homeostasis further induces the F-actin stress fiber derangement and cytoskeleton reorganization. Our data indicate novel mechanistic insights into the role of intracellular calcium homeostasis in regulating trophoblast cell motility, and they imply that InsP₃R-modulated calcium signaling is associated with this process. Together, our study indicates that the dysregulation of SEC5 expression may be involved in the impaired migratory and invasive capacities of the trophoblast, and it may serve as a therapeutic target in placenta-associated diseases.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-19-0088>.

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

W-W G, L Y, X Z and J W designed the study and experiments. W-W G, L Y, X-X Z, M L, and Q Y carried out all the experiments. Y G and H X collected the placenta tissues. All authors critically revised the manuscript and approved the final version.

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