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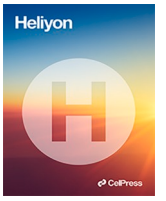

Research article

CXC chemokine receptor type 5 may induce trophoblast dysfunction and participate in the processes of unexplained missed abortion, wherein *p*-ERK and interleukin-6 may be involved

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ABSTRACT

Chemokines regulate the trophoblast dysfunction involved in the occurrence and development of pathological pregnancy, including missed abortions. In particular, CXC chemokine receptor type 5 mediates cell proliferation, migration, and inflammation; nonetheless, its role in missed abortions remains unclear. This study aimed to examine the expression of CXC chemokine receptor type 5 in missed abortions and to investigate the effects of CXC chemokine receptor type 5 on the biological behaviour of trophoblasts, as well as the underlying mechanisms. Our results indicated that CXC chemokine receptor type 5 was upregulated in the villi of women who experienced unexplained missed abortions, as compared with those who had normal pregnancies. CXC chemokine receptor type 5 inhibited the proliferation and migration of human first-trimester trophoblast/simian virus cells but promoted cell apoptosis. With respect to its mechanisms, CXC chemokine receptor type 5 activated the extracellular signal-regulated protein kinase 1/2 signalling pathway and upregulated the secretion of interleukin-6; however, it had no effect on the secretion of tumour necrosis factor-α. In conclusion, our findings suggest that CXC chemokine receptor type 5 induces trophoblast dysfunction and participates in the processes of unexplained missed abortions, wherein *p*-ERK and interleukin-6 may be involved.

1. Introduction

Missed abortion (MA) is generally defined as the presence of a dead embryo or foetus in the uterine cavity that is not spontaneously discharged in time. In China, the incidence of MA is approximately 15 % and is increasing every year. Currently, environmental factors, genital malformations, endocrine disorders, chromosomal diseases, immune system diseases, and infections have all been recognized

Abbreviations: CCK-8, Cell Counting Kit-8; CXCL13, CXC chemokine ligand 13; CXCR5, CXC chemokine receptor type 5; ELISA, enzyme-linked immunosorbent assay; ERK1/2, extracellular signal-regulated protein kinase 1/2; HE, Haematoxylin–eosin; HTR-8/SVneo, human first-trimester trophoblast/simian virus; IHC, immunohistochemistry; IL-6, interleukin-6; MA, missed abortion; PKB, protein kinase B; PVDF, polyvinylidene difluoride; qRT-PCR, quantitative real-time polymerase chain reaction; STAT3, signal transducer and activator of transcription 3; TNF-α, tumour necrosis factor-α.

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as risk factors for MA [1]; nevertheless, there are still some unknown causes. Therefore, clarifying the potential biological mechanisms of MA is of considerable importance for the treatment of this disease.

The maintenance of a normal pregnancy depends on the synchronous development of the blastocyst and endometrium and requires a highly coordinated two-way signal transmission between the mother and foetus. Trophoblasts play important roles in this process, including the synthesis and secretion of multiple hormones and cytokines, nutrient exchange, endocrine and metabolic activities, and invasion of the endometrium, uterine blood vessels, and glands [2]. Abnormal trophoblast function leads to pathological pregnancy, including abortion, preeclampsia, and foetal growth restriction [3].

Chemokines, which comprise small molecules with a similar structure, activate various signalling pathways through membrane-bound G-protein-coupled receptors [4] and participate in physiological activities, such as cell immunity, inflammation, growth, and development [5]. Furthermore, chemokines are expressed at the maternal–foetal interface and are involved not only in the maintenance of a normal pregnancy but also in the occurrence and development of a pathological pregnancy [6,7].

CXC chemokine receptor type 5 (CXCR5) is the ~~only~~ receptor for CXC chemokine ligand 13 (CXCL13), which is highly expressed in mature B lymphocytes, follicle-assisted T cells, and migrating skin-derived dendritic cells [8]. Stimulated by CXCL13, G proteins dissociate from CXCR5 and activate the downstream signalling pathways to mediate various reactions, such as cell proliferation, intracellular calcium increase, invasion, migration, and gene transcription [9]. Thus, CXCR5 is widely involved in the occurrence and development of numerous conditions, including cancer, infectious diseases, idiopathic pulmonary fibrosis, transplant rejection, neuropathic pain, and autoimmune diseases [10]. CXCR5 is located in trophoblasts during the first trimester, and its expression level in the villi has been reported to be higher among patients who had experienced recurrent abortion than among those who had a normal pregnancy [11]. Hence, we assume that CXCR5 could be involved in the occurrence of abortion; nonetheless, the underlying mechanisms remain unclear.

The present study aimed to examine the expression of CXCR5 in the villi of women who had MA, to investigate the effects of CXCR5 on the biological behaviour of human first-trimester trophoblast/simian virus (HTR-8/SVneo) cells, and to explore its underlying molecular mechanisms.

2. Materials and methods

2.1. Clinical samples

Patients were recruited at Hebei General Hospital (Shijiazhuang, China) from January 2021 to September 2021. All experiments and the use of villi were approved by the Ethics Committee of Hebei General Hospital (approval no.: 2021214) and were performed in accordance with the World Medical Association's Code of Ethics (Declaration of Helsinki). All participants signed written informed consent prior to any experiment. And consent to publish the data of participants was acquired.

In total, 21 women who had a normal pregnancy and 21 MA women who underwent induced abortion were randomly selected. Women who had ultrasound-confirmed pregnancy with 5–12 weeks of gestational age and who had prior regular menstruation were considered eligible for this study. The exclusion criteria were as follows: (i) multiple pregnancies, (ii) use of hormonal medication, and (iii) known risk factors for miscarriage such as infectious diseases, chromosomal abnormalities, endocrine diseases, autoimmune diseases, and chronic illnesses. The control group consisted of women with healthy pregnancies who underwent induced abortion in the early gestational stage because of family planning or other non-health-related reasons and who exhibited no signs of miscarriage during pregnancy or had no history of miscarriage or stillbirth. MA was diagnosed based on the following criteria established by the Society of Radiologists in Ultrasound [12]: crown–rump length $\geq$7 mm, no detectable foetal heartbeat, average gestational sac diameter $\geq$25 mm, and no visible foetal pole.

All patients underwent vacuum aspiration under intravenous anaesthesia to terminate the pregnancy. Fresh tissue samples were obtained and repeatedly rinsed in sterile physiological saline to remove the blood. The villi and decidua were separated, and physiological saline in villus tissues was aspirated using a sterile filter paper. Villus tissues were divided into two parts: one part was fixed in 4 % paraformaldehyde, whereas the other part was quickly placed in a liquid nitrogen tank after being placed in a ribozyme-free cryopreservation tube. All of these aforementioned operations were completed within 15 min after separation. Specimens fixed in 4 % paraformaldehyde were stored at room temperature, whereas specimens in the liquid nitrogen tank were stored in a −80 °C refrigerator.

2.2. Cell culture and transfection

HTR-8/SVneo cells (RRID: CVCL_7162) (Zhong Qiao Xin Zhou Biotechnology, Shanghai, China) were certified using Short Tandem

Table 1
Primer sequences.

Gene	Primers	Sequences (5′ to 3′)
CXCR5	Forward	CACGTTGCACCTTCTCCCAA
	Reverse	GGAATCCCGCCACATGGTAG
β-actin	Forward	CATGTACGTTGCTATCCAGGC
	Reverse	CTCCTTAATGTCACGCACGAT

Repeat analysis and were routinely tested for mycoplasma contamination. HTR-8/SVneo cells were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10 % foetal bovine serum (ExCell Bio, Shanghai, China) and 1 % penicillin-streptomycin (Solarbio, Beijing, China) in an incubator containing 5 % CO_2 at 37 °C.

At cell confluence of approximately 70–90 %, the medium was replaced with basic medium containing 50 nM si-CXCR5 (RiboBio, Guangzhou, China) blended with 7.5 μL Lipofectamine 2000 Reagent (Thermo Fisher Scientific, USA) or basic medium containing 2.5 μg CXCR5 overexpression pcDNA3.1 vector (YouBio, Changsha, China) blended with 7.5 μL Lipofectamine 2000 Reagent. The respective control group was transfected with the same dose of empty vector. Subsequently, the cells were incubated in a complete medium after transfection for 6 h. Transfection efficiency was determined by quantitative real-time polymerase chain reaction (qRT-PCR) after 24 h and western blotting after 48 h.

2.3. qRT-PCR

Total RNA was extracted from the villi or HTR-8/SVneo cells transfected for 24 h using the RNA Easy Fast Tissue/Cell Kit (TIANGEN, Beijing, China). The concentration and purity of RNA were determined using NanoDrop 2000 (Thermo Fisher Scientific, MA, USA). Reverse transcription of RNA to cDNA was conducted with FastKing gDNA Dispelling RT SuperMix (TIANGEN, Beijing). qRT-PCR was performed using SuperReal PreMix Plus (SYBR Green) (TIANGEN, Beijing) on a real-time fluorescence quantitative PCR (Life Technologies Holdings Pte Ltd., Singapore). The relative expression levels of CXCR5 mRNA were determined using the $2^{-\Delta\Delta CT}$ method, with β-actin as the reference control. The primers were synthesised by General Biosystems (Anhui, China), and the sequences are listed in Table 1.

2.4. Western blotting

Total protein of villi and HTR-8/SVneo cells transfected for 48 h was extracted with RIPA lysis buffer (Solarbio, Beijing, China) supplemented with protease inhibitor (Solarbio, Beijing, China) and phosphatase inhibitor (Solarbio, Beijing, China). The total protein concentration was determined using a BCA assay kit (Solarbio, Beijing, China). Equal amounts of proteins were separated through polyacrylamide gel electrophoresis (Yeasen, Shanghai, China) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, USA). After blocking in 5 % non-fat milk (Solarbio, Beijing, China) for 2 h at room temperature, the PVDF membrane was incubated with 1:1500 diluted rabbit anti-human CXCR5 antibody (Affinity, USA), 1:1500 diluted rabbit anti-human *p*-ERK1/2 antibody (Affinity, USA), or 1:2000 diluted rabbit anti-human β-actin antibody (Servicebio, Wuhan, China) overnight at 4 °C and subsequently incubated with 1:5000 diluted goat anti-rabbit secondary antibody (Affinity, USA) for 1 h at room temperature. Protein blots were visualised using an enzyme-linked chemiluminescence solution (4A BIOTECH, Beijing, China). Densitometric analysis was conducted using ImageJ software (National Institutes of Health, USA), with β-actin for normalisation.

2.5. Haematoxylin–eosin (HE) staining and immunohistochemistry (IHC)

HE staining and IHC analysis were performed by Servicebio (Wuhan, China). The experiments were carried out using an HE staining kit (Servicebio, Wuhan, China) with 1:50 diluted rabbit anti-human CXCR5 antibody (Affinity, USA) and 1:200 diluted HRP-labelled goat anti-rabbit antibody (Abbkine, Wuhan, China). Sections were examined under a microscope (Nikon, Tokyo, Japan). DAB IHC was quantified using the average optical density value analysed by Aipathwell software (Hongrui Biological Company, Guangzhou, China).

2.6. Cell counting Kit-8 (CCK-8) assay

After transfection for 24 h, HTR-8/SVneo cells were inoculated into 96-well plates (5000 cells/well) and subsequently cultured in an incubator for 0, 24, 48, and 72 h. Following the addition of 10 μL CCK-8 reagent (APExBIO, USA) into each well, the cells were cultured for another 4 h. The optical density at 450 nm was measured to construct proliferation curves.

Table 2
Clinical characteristics of the study participants.

Groups	Maternal age (years)	Gestational age (days)	Body mass index (kg/m^2)	Previous pregnancy history	Previous birth history
Normal group (n = 21)	28.90 ± 6.06	49.86 ± 5.52	22.21 ± 3.68	2 [1; 3]	1 [0; 1]
MA group (n = 21)	31.24 ± 4.25	52.14 ± 5.71	23.29 ± 2.86	2 [1; 3]	0 [0; 1]
t/Z value	−1.445	−1.319	−1.064	−0.340	−1.263
P-value	0.157[a]	0.195[a]	0.294[a]	0.734[b]	0.207[b]

Continuous variables are presented as mean ± standard deviation, and enumeration data are expressed as median [1st quantile; 3rd quantile].
[a] Independent samples *t*-test.
[b] Mann–Whitney *U* test. Statistical significance was set at $P < 0.05$.

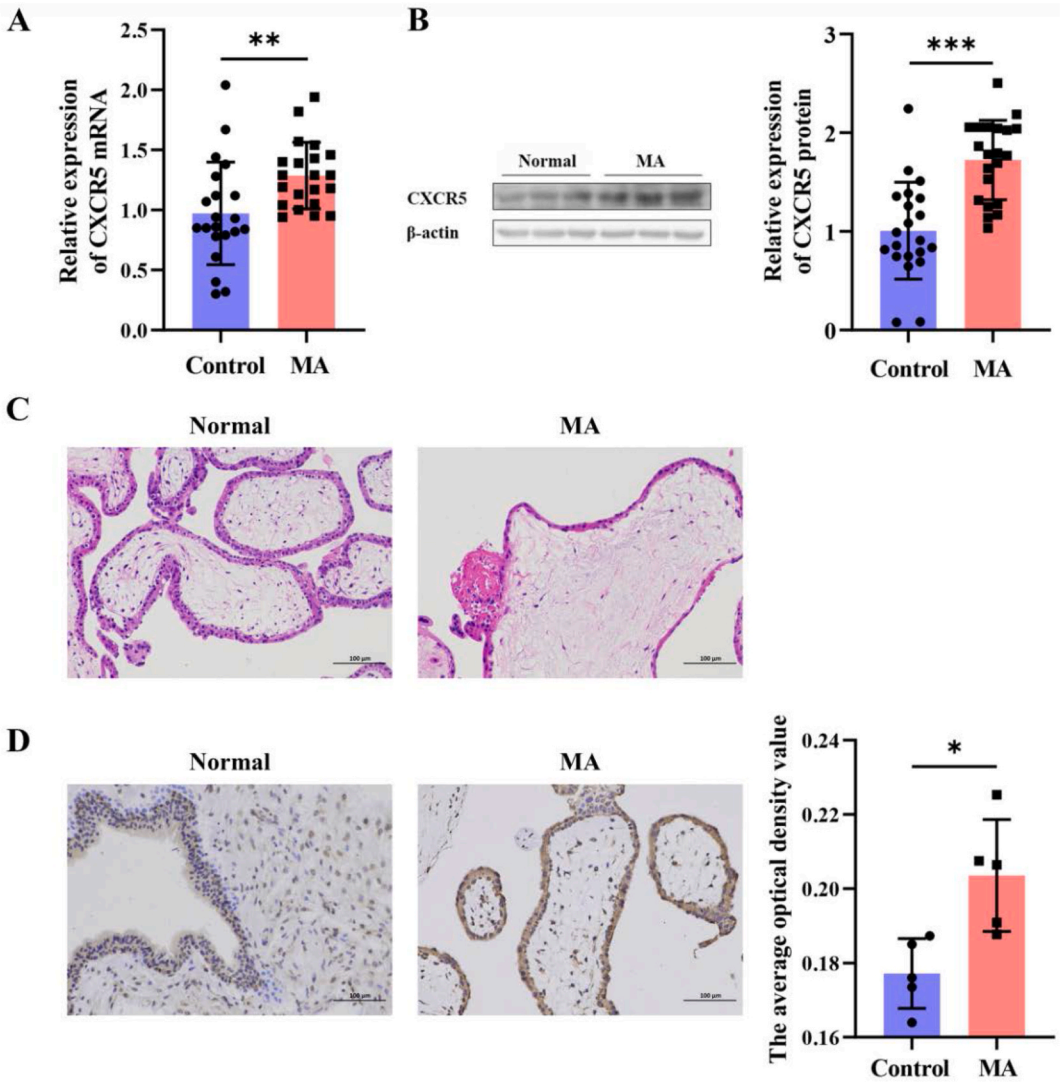

Fig. 1. CXCR5 expression was upregulated in the villi of unexplained MA. (A, B) mRNA and protein levels of CXCR5 were measured by qRT-PCR (A) and western blotting (B) in villi from women with normal pregnancy (n = 21) or unexplained MA (n = 21). (C) HE staining was used to observe the pathological morphology at a magnification of 200X. (D) CXCR5 protein was detected and located by IHC analysis in villi from women with normal pregnancy (n = 5) or unexplained MA (n = 5) at a magnification of 200X. Scale bar (C, D) = 100 μm *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

2.7. Transwell assay

After transfection for 24 h, HTR-8/SVneo cells (20×10^4/well) were resuspended in 150 μL serum-free medium and inoculated into the upper chamber of Transwell (Corning, USA); the 600 μL complete medium containing 20 % foetal bovine serum was added into the lower chamber. After culturing in an incubator for 24 h, the cells on the upper side of the membrane were wiped with cotton swabs, whereas the cells penetrating the membrane were fixed with 4 % paraformaldehyde (Solarbio, Beijing, China) for 30 min and then stained with 0.1 % crystal violet (Solarbio, Beijing, China) for 10 min. Migratory HTR-8/SVneo cells were observed and counted for five fields using an optical microscope (Leica, Germany).

2.8. Wound healing assay

HTR-8/SVneo cells (5×10^5/well) were seeded into 6-well plates for transfection. After transfection for 24 h, a cell wound was created using a 200 μL pipette tip. The cells were washed three times with phosphate-buffered saline, with a serum-free medium being added for culture. The wound healing area was measured under a microscope (Leica, Wetzlar, Germany) at 0 h and 24 h.

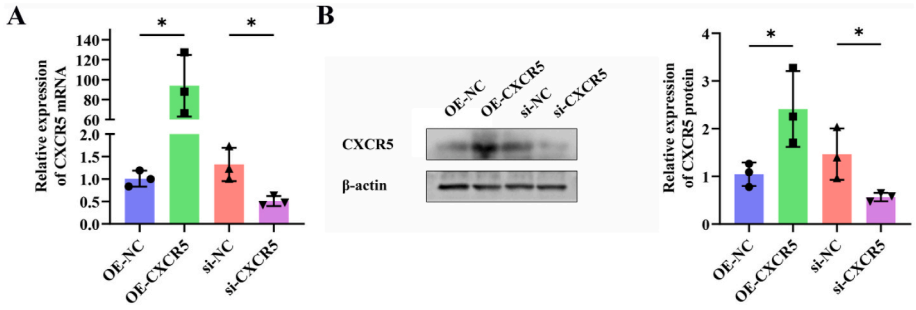

Fig. 2. Transfection efficiency was confirmed by qRT-PCR and western blotting. (A) CXCR5 mRNA levels in HTR-8/SVneo cells transfected for 24h. (B) CXCR5 protein levels in HTR-8/SVneo cells transfected for 48h. β-actin was used as reference control. The results are represented as mean ± standard deviation of at least three independent experiments. OE-NC: negative control cells transfected with empty vector of plasmid. OE-CXCR5: cells transfected with CXCR5 overexpression pcDNA3.1 vector. si-NC: negative control cells transfected with empty siRNA. si-CXCR5: cells transfected with siRNA targeting CXCR5. *$P < 0.05$.

2.9. Flow cytometry

The apoptosis rate was analysed using the Annexin V-FITC/PI Apoptosis Kit (Multi Sciences, Hangzhou, China). After transfection for 48 h, HTR-8/SVneo cells were centrifuged at 1000 rpm for 5 min, washed with phosphate-buffered saline, and resuspended in 500 μL 1 × binding buffer. Subsequently, 5 μL Annexin V-FITC and 10 μL PI were added, and the cell suspension was detected by flow cytometry (BD, Suzhou, China) after being placed in darkness for 5 min at room temperature.

2.10. Enzyme-linked immunosorbent assay (ELISA)

Concentrations of interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) in the supernatant of HTR-8/SVneo cells transfected for 48 h were determined using an ELISA kit (4A BIOTECH, Beijing).

2.11. Statistical analysis

Statistical analysis was performed using SPSS version 21.0 and GraphPad Prism version 8.0.2. Normally distributed continuous variables were presented as means ± standard deviations and analysed using the unpaired *t*-test; furthermore, repeated measurement data were compared using repeated-measures analysis of variance with post hoc Bonferroni correction. Non-normally distributed data were expressed as median [1st quantile; 3rd quantile] and analysed using non-parametric tests. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Clinical features of the study participants

Table 2 summarises the clinical characteristics of the two groups of study participants. Women who experienced unexplained MA and those who had normal pregnancy showed no statistically significant differences with respect to age, gestational age, body mass index, previous pregnancy history, and previous birth history ($P > 0.05$, Table 2).

3.2. Expression of CXCR5 was upregulated in the villi of women who had unexplained MA

For the analysis of the CXCR5 expression in women who experienced unexplained MA, the mRNA and protein expression levels of CXCR5 in the villi were measured using qRT-PCR, western blotting, and IHC analysis. CXCR5 was mainly located in the cell membranes and cytoplasms of syncytiotrophoblasts and cytotrophoblasts, respectively (Fig. 1D). The expression levels of CXCR5 mRNA were significantly increased in the villi of women who had unexplained MA, as compared with those of women who had normal pregnancy (Fig. 1A), which was consistent with the results for the protein levels (Fig. 1B–D). HE staining indicated that when compared with those of women with normal pregnancy, the villi of women with unexplained MA showed abnormal morphology, including an irregular shape, discontinuous trophoblast distribution, single-layer arrangement of cells, interstitial oedema with loose structure, and multiple necrotic fragments (Fig. 1C).

3.3. CXCR5 suppressed the proliferation and migration of HTR-8/SVneo cells but promoted cell apoptosis

The effects of CXCR5 on the biological behaviour of HTR-8/SVneo cells were further investigated. Both qRT-PCR and western blotting confirmed the downregulation or overexpression of CXCR5 in HTR-8/SVneo cells (Fig. 2A and B). Analysis of the role of

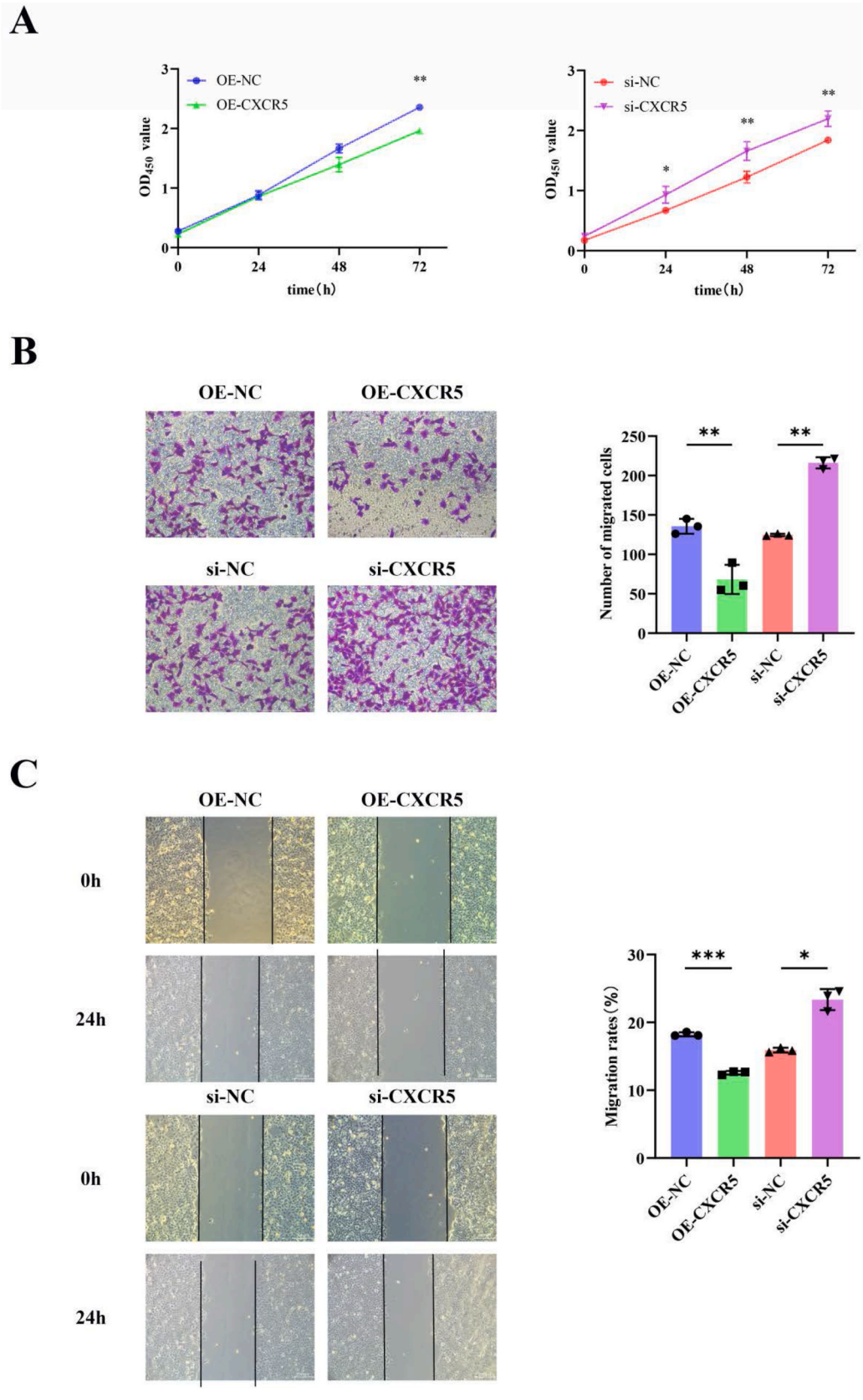

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Fig. 3. CXCR5 suppresses proliferation and migration of HTR-8/SVneo cells. (A) Cell proliferation was confirmed by CCK-8. (B, C) Cell migration properties were studied by Transwell at a magnification of 200×(B) and wound healing assay at a magnification of 100X(C). The results are represented as mean ± standard deviation of at least three independent experiments. OE-NC: negative control cells transfected with empty vector of plasmid. OE-CXCR5: cells transfected with CXCR5 overexpression pcDNA3.1 vector. si-NC: negative control cells transfected with empty siRNA. si-CXCR5: cells transfected with siRNA targeting CXCR5. Scale bar = 200 μm $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

CXCR5 in cell proliferation using the CCK-8 assay revealed that cell proliferation was inhibited by CXCR5 overexpression but was enhanced by CXCR5 downregulation (Fig. 3A). The effects of CXCR5 on cell migration were studied using Transwell and wound healing assays. CXCR5 overexpression significantly inhibited the migration ability of HTR-8/SVneo cells, whereas CXCR5 downregulation increased the migration ability of these cells (Fig. 3B). The wound healing assay further confirmed that CXCR5 overexpression disrupted the wound healing ability of the cells, whereas CXCR5 knockdown enhanced their wound healing ability (Fig. 3C). Lastly, the effects of CXCR5 on the cell apoptosis rate were determined by flow cytometry. The apoptosis rate increased when CXCR5 was highly expressed but decreased when CXCR5 was knocked down (Fig. 4).

Overall, these results indicated that CXCR5 inhibited the proliferation and migration of HTR-8/SVneo cells but promoted cell apoptosis, suggesting that CXCR5 might play an important role in trophoblast dysfunction.

3.4. CXCR5 activated the extracellular signal-regulated protein kinase 1/2 (ERK1/2) pathway and upregulated the secretion of IL-6 rather than that of TNF-α in HTR-8/SVneo cells

The ERK1/2 pathway has been proven to be one of the important signalling pathways involved in trophoblast function. IL-6 and TNF-α are downstream targets of the ERK1/2 signalling pathway and participate in trophoblastic inflammation. Therefore, western blotting was performed to investigate whether abnormal CXCR5 expression would affect the phosphorylation of ERK1/2 protein in HTR-8/SVneo cells. The results showed that the expression level of *p*-ERK1/2 was increased in cells with CXCR5 overexpression but decreased in cells with low CXCR5 expression (Fig. 5A). Subsequently, ELISA was employed to detect the concentrations of IL-6 and TNF-α in the supernatant. The results indicated that the secretion of IL-6 was promoted after CXCR5 was upregulated but was suppressed after CXCR5 was downregulated (Fig. 5B). In contrast, the CXCR5 expression did not interfere with the secretion of TNF-α (Fig. 5C). Overall, these data suggest that CXCR5 causes trophoblastic inflammation by activating the ERK1/2 pathway and upregulating the secretion of IL-6 rather than TNF-α.

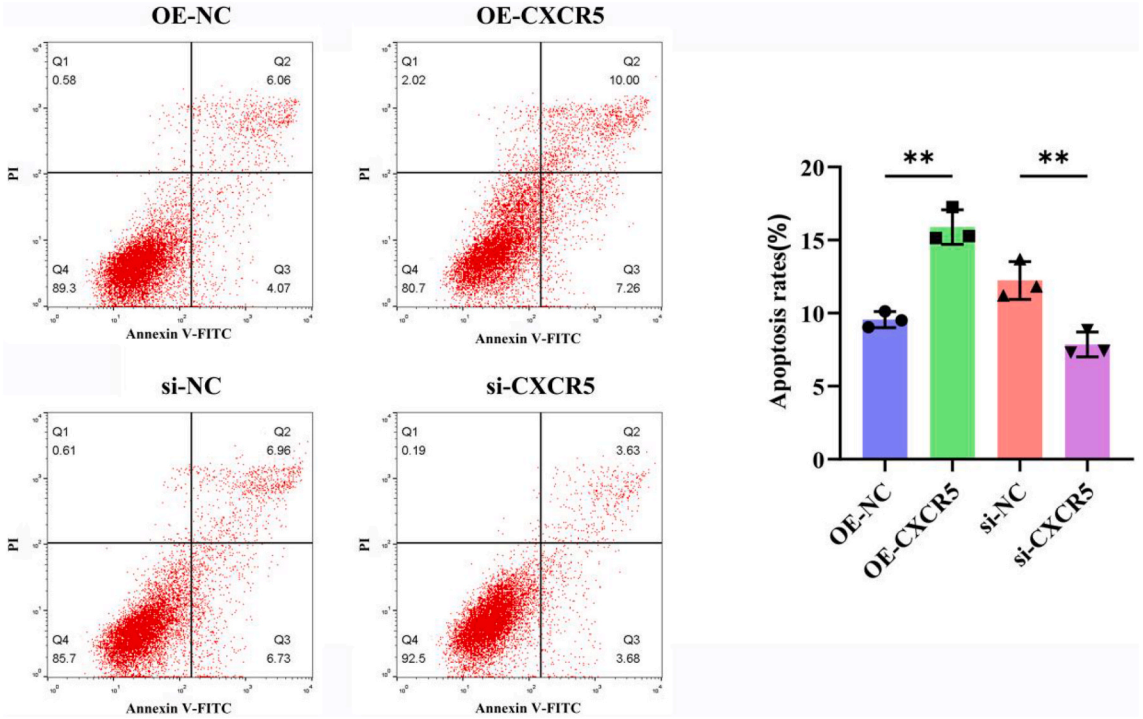

Fig. 4. CXCR5 promotes apoptosis of HTR-8/SVneo cells. The results are represented as mean ± standard deviation of at least three independent experiments. OE-NC: negative control cells transfected with empty vector of plasmid. OE-CXCR5: cells transfected with CXCR5 overexpression pcDNA3.1 vector. si-NC: negative control cells transfected with empty siRNA. si-CXCR5: cells transfected with siRNA targeting CXCR5. $^{**}P < 0.01$.

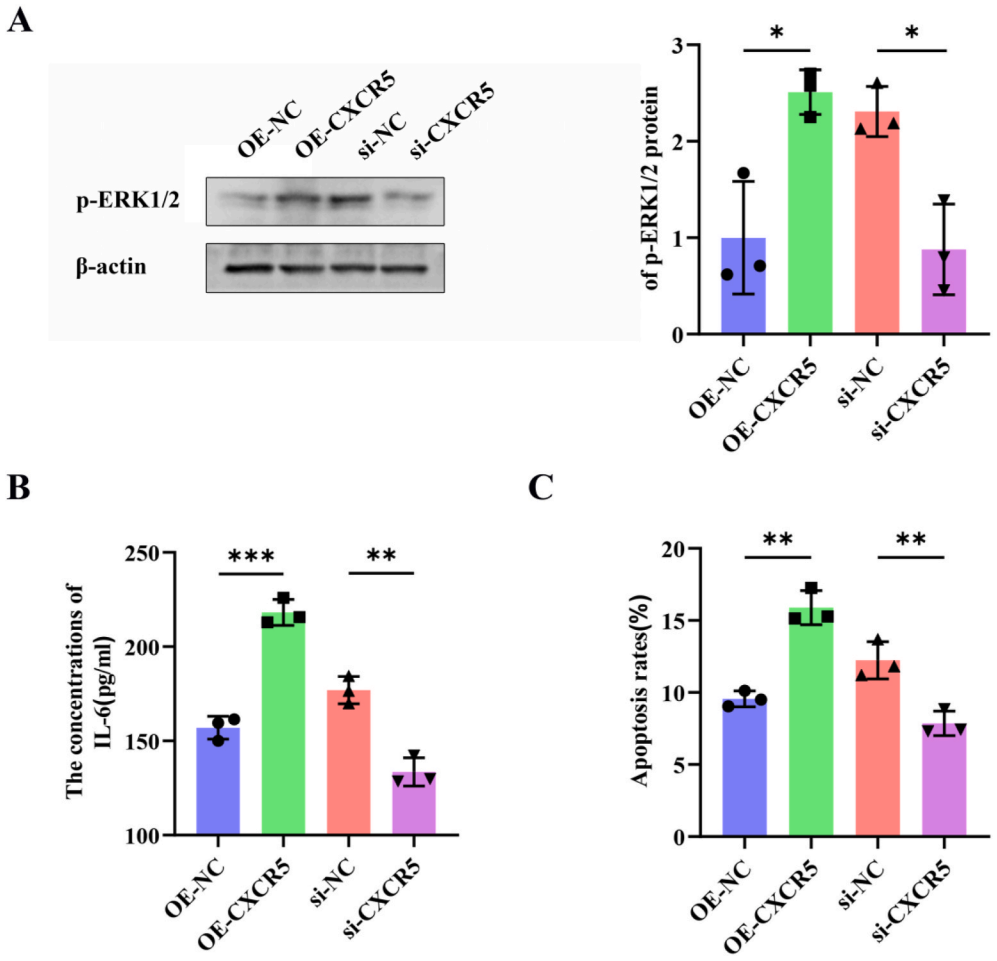

Fig. 5. CXCR5 upregulates the secretion of IL-6 rather than that of TNF-α via ERK1/2 pathway. (A) *p*-ERK1/2 protein in HTR-8/SVneo cells was detected by western blotting. (B) The concentration of IL-6 in the supernatant of HTR-8/SVneo cells was measured by ELISA. (C) The concentration of TNF-α in the supernatant of HTR-8/SVneo cells was measured by ELISA. The results are represented as mean ± standard deviation of at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

4. Discussion

The maintenance of early pregnancy relies on the normal occurrence of embryo implantation. Trophoblasts play an important role in immune regulation, cell adhesion and invasion, and vascular remodelling [13,14]. Trophoblast dysfunction can lead to miscarriage, preeclampsia, preterm delivery, placenta accreta, and other pathological pregnancies [15–18]. Compared to those in women with normal pregnancy, the trophoblasts in MA patients have higher levels of oxidative stress and apoptosis rates; at the same time, their capacity for proliferation, viability, and migration is suppressed [19–22]. These findings suggest that the occurrence and development of MA are associated with multiple functional abnormalities in trophoblasts; however, the specific regulatory mechanisms have not been fully elucidated.

Our observation of histological sections revealed that the morphology of villi from MA patients was abnormal, which is consistent with the findings of Jing et al. [23]. We speculate that an abnormal trophoblast structure may be associated with a disturbance in biological or endocrine function, leading to miscarriage. Trophoblasts secrete various cytokines and hormones involved in the maintenance of pregnancy. Cytokines, hormones, and immune cells can also regulate the trophoblast function at the maternal–foetal interface [24,25]. CXCR5, a chemokine receptor, is the ~~only~~ receptor of CXCL13. The CXCL13/CXCR5 axis has been confirmed to be involved in the occurrence and development of several diseases. In this study, by using qRT-PCR, western blotting, and IHC analysis, we determined that the CXCR5 mRNA and protein expression levels were higher in the villi of patients who experienced MA than in those of women who had a normal pregnancy. Through IHC analysis, we also confirmed that CXCR5 was mainly expressed in the cell membranes and cytoplasms of trophoblasts, which is consistent with the findings of Gong et al. [11], who also reported that the expression level of CXCR5 was higher in the villi of patients who experienced recurrent miscarriages than in those of women who had normal pregnancies [11]. The aforementioned results indicated that CXCR5 might participate in the development and progression of MA.

The CXCL13/CXCR5 axis plays a role in tumour growth via [26] (i) the induction of cell proliferation and the inhibition of apoptosis to directly regulate tumour growth, (ii) the promotion of cell invasion and motility, and (iii) the indirect regulation of cell growth through immune cell modulation. Considering that the biological behaviour of trophoblasts is similar to that of tumour cells, we examined the effects of CXCR5 on the trophoblast function and concluded that CXCR5 inhibited the proliferation and migration of HTR-8/SVneo cells but promoted cell apoptosis. However, the regulation of trophoblast function by CXCR5 is opposite to that in tumours, indicating the existence of tissue heterogeneity with respect to CXCR5. We speculate that CXCR5 exerts differential regulatory effects on tumour cells and trophoblasts by activating different signalling pathways.

As an original member of the mitogen-activated protein kinase family, ERK can be affected by extracellular stimuli such as cellular stress and inflammation. It is then rapidly phosphorylated to mediate the cell cycle and to regulate cell adhesion, proliferation, and apoptosis, as well as RNA processing and protein synthesis [27]. Therefore, ERK plays an important role in physiological regulation, including growth and development, cell homeostasis, and diseases [27]. ERK has been confirmed to be the downstream signalling pathway of CXCL13/CXCR5 in various diseases. In breast cancer, CXCL13/CXCR5 activates ERK to regulate the production of the inflammatory cytokines IL-1β and TNF, which are involved in tumour growth and progression [28]. Blocking the CXCL13/CXCR5 signalling axis reduces the *p*-ERK/ERK levels, thereby upregulating the cyclin D1 expression, downregulating the caspase-9 expression, promoting apoptosis, and consequently inhibiting breast cancer cell proliferation [29]. In mouse spinal cord, CXCL13 was found to promote the production of TNF-α and IL-6 by binding to CXCR5, subsequently activating ERK, protein kinase B (PKB), and signal transducer and activator of transcription 3 (STAT3) signalling pathways, and finally mediating the development of diabetic neuralgia [30]. Studies using rat models of spinal cord ischemia–reperfusion also showed that CXCR5 inhibition reduced the expression of *p*-ERK and inflammatory cytokines, thereby protecting rats from spinal cord ischemia–reperfusion injury [31]. In our experiments, the expression level of *p*-ERK1/2 was increased in HTR-8/SVneo cells after CXCR5 expression was upregulated but was conversely decreased when CXCR5 expression was downregulated. A significant difference between OE-NC and si-NC was observed, which may be attributed to the different pcDNA and siRNA transfection vectors. We believe that CXCR5 could activate the ERK1/2 signalling pathway in trophoblasts. However, there exists no consensus on the effects of ERK on trophoblasts. Previous studies reported that the activation of the ERK1/2 signalling pathway regulated the expression of matrix metalloproteinase 9, vimentin, and E-cadherin, which promoted trophoblast proliferation, migration, invasion, and epithelial-mesenchymal transition [32,33]. However, studies on trophoblastic inflammation observed that upregulated ERK1/2 phosphorylation increased the secretion of IL-6 and IL-8, resulting in the attenuation of trophoblast migration and invasion [34–36]. ERK1/2 phosphorylation might regulate various downstream molecules and exert different effects on trophoblast proliferation, migration, invasion, and apoptosis.

IL-6 is present in both syncytiotrophoblasts and extravillous trophoblasts [37], whereas the IL-6 receptor is found in the endometrium, decidua, and trophoblasts during embryo implantation and placentation. During the first trimester of pregnancy and labour, moderate TNF-α levels mediate proinflammatory immune responses and promote embryo transplantation and parturition. During the second and third trimesters of pregnancy, the inflammatory response is inhibited, contributing to embryonic development [38]. An increase in proinflammatory cytokines, including TNF-α, IL-6 and IL-8, may activate inflammatory responses, impair trophoblast function, and result in pregnancy complications, such as infertility, miscarriage, and preeclampsia [39–41]. Scholars have previously detected a significant upregulation of IL-6 and IL-8 expression in villi from recurrent spontaneous abortions [39,42]. In JEG-3 cells, IL-6 expression is promoted by the phosphorylation of the ERK1/2 pathway [43]. Increased IL-6 secretion leads to diminished cell migration [44]. In our study, CXCR5 upregulated the secretion of IL-6 in HTR-8/SVneo cells, but the secretion of TNF-α was not significantly modulated by CXCR5.

This study has some limitations. Physiological activities in humans are complex. However, we conducted only *in vitro* cell experiments and only discussed the effect of CXCR5 on IL-6 and TNF-α secretion. Follow-up studies are therefore required to verify the role of CXCR5 in MA and to investigate its underlying molecular mechanisms in animal models. The production and secretion of other classical cytokines, such as IL-10 and IL-1β, also remain to be investigated. Furthermore, whether CXCL13 regulates trophoblast function via CXCR5 and is involved in MA requires exploration and will be the next focus of our research, where we will investigate the effect of CXCL13 stimulation *in vitro* assays.

In conclusion, CXCR5 may induce trophoblast dysfunction and participate in MA, in which *p*-ERK and IL-6 may be involved.

Funding

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Ethical approval

All experiments and villi used were approved by the Ethics Committee of Hebei General Hospital in 2021(approval no.: 2021214). Each participant has consented to participate and publish the data.

Data availability statement

The raw data cannot be shared at this time as the data also forms part of an ongoing study.

CRediT authorship contribution statement

Yanan Zhi: Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Pingping Zhang:** Writing – review & editing, Validation, Formal analysis. **Yan Luo:** Visualization, Supervision. **Yanmei Sun:** Writing – review & editing, Validation, Software. **Juan Li:** Validation, Supervision, Resources, Data curation. **Mingming Zhang:** Visualization, Supervision, Resources, Investigation. **Yali Li:** Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31465.

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