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Original Article

Dysregulation of CD44v6 may lead to recurrent spontaneous abortion by inhibiting the proliferation and migration of trophoblast cells

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Abstract: In the establishment and continuation of a successful pregnancy, the proliferation, migration, and invasion of trophoblast cells play essential roles. Impaired trophoblast function has been reported to be involved in recurrent spontaneous abortion (RSA) but the potential mechanisms are still unknown. CD44 variant domain 6 (CD44v6) is a transmembrane glycoprotein that has been known for decades to be expressed in the placenta. In this study, we investigated the effect of CD44v6 on proliferation and migration of trophoblast cells. Our results show that CD44v6 expression in the villi is lower in RSA patients than in women with normal pregnancies. Furthermore, downregulation of CD44v6 leads to a reduction in proliferation and migration in the human trophoblast cell line HTR-8/SVneo, along with decreased expression and nuclear translocation of NF-κB. These results suggest that decreased expression of CD44v6 may cause miscarriage by downregulating trophoblast cell proliferation via the NF-κB pathway.

Keywords: CD44v6, NF-κB, trophoblast cell, recurrent spontaneous abortion, proliferation, invasion, HTR-8/SVneo cell

Introduction

Recurrent spontaneous abortion (RSA) is defined as two or more failed pregnancies [1] and is also called recurrent pregnancy loss (RPL), recurrent miscarriage (RM), idiopathic recurrent miscarriage (IRM), or habitual abortion. RSA affects approximately 1% of fertile couples worldwide [2]. Over a long period of time, many studies have been conducted to determine the causes of RSA and multiple factors have been confirmed including parental chromosomal abnormalities, anatomical malformations of the uterus, autoimmune diseases, endocrine disorders, and infections but the cause of RSA remains unknown in approximately half of all cases [3]. Because of its high incidence rate and poor cure rate, many patients have suffered a great deal.

Trophoblast cells play an essential role in establishing early pregnancy and only when trophoblast invasion into the uterus decidua is accomplished can a successful pregnancy be established. In this process, placental cytotrophoblast cells must undergo a change similar to the transformation of the epithelial mesenchymal transition (EMT), that is, from closely connected to migratory. Functionally impaired trophoblasts may give rise to injuries of uterine spiral artery reconstruction and, thus, pregnancy-related complications such as recurrent miscarriage and pre-eclampsia may occur [4, 5].

The adhesion molecule CD44 is a type of transmembrane glycoprotein that plays an important role in cell-cell and cell-matrix adhesion, hematopoiesis, lymphocyte homing, and tumor dissemination [6, 7]. Its genes are composed of 10 constitutive exons and 10 variant exons separated by introns of different lengths. CD44 isoforms consisting only of constitutive exons are called standard CD44 (CD44s) and the other isoforms, originating from selective splicing of variant exons, are called CD44vs. In total, 10 variants have been confirmed and named CD44v1-10. Of these, CD44v6 occupies an important position as it is associated with the

tumorigenic potential of many kinds of human neoplasms specifically tumor invasion, progression, and metastasis abilities [8-12].

Several decades ago, CD44v6 was confirmed to be expressed in the placenta [13]. Zöller M confirmed that when female mice received administration of anti-CD44v6 antibodies, beginning at the time of mating, the probability of conception decreased significantly, even when the mating period was extended to 4 days. This suggested that CD44v6 may participate in RSA development [14]. However, the functional mechanism of CD44v6 in trophoblast and pregnancy outcomes has not yet been determined.

NF-κB is a positive transcription factor that regulates multiple gene expression profiles and physiological actions. The most important and conserved function of NF-κB lies in the mediation of immunity and inflammatory response. In addition, NF-κB also participates in cell adhesion, differentiation, proliferation, and inhibition of apoptosis. Activation of NF-κB has been reported to take part in diseases such as cancer, arthritis, and chronic inflammation [15, 16]. There are five members of the NF-κB family namely, p65(RelA), RelB, c-Rel, NF-κB1, and NF-κB2 [17]. Each member constitutes homogeneous or heterogeneous dimers and has a common Rel homology domain, which is necessary for dimerization and combination with the corresponding DNA elements [18]. Under resting conditions, NF-κB dimers are combined with IκB proteins or the precursor proteins p100 and p105 in the cytoplasm. The activation of NF-κB can be translocated to the nucleus and activate target genes [19, 20]. A previous report confirmed the association of NFκB1 gene variants with risk of idiopathic recurrent miscarriages (IRM) [21].

In this study, we evaluated CD44v6 and NF-κB expression in villi from RSA and normal early pregnancy. Then, the cell line HTR-8/SVneo was used to investigate the function of trophoblasts. After transfecting the cell line with CD44v6-specific siRNA, the impact of CD44v6 on cell proliferation and migration was evaluated by cloning experiments and Transwell migration analyses. The results show that expression of CD44v6 and NF-κB is decreased in RSA compared with normal pregnancy. Proliferation and invasion ability of trophoblast cells also declined, as did NF-κB nuclear translocation.

Materials and methods

Sample collection

This study was designed as a prospective controlled study. The study was conducted from March 2015 to March 2016 in the Outpatient Operation Room of the Department of Gynaecology and Obstetrics of Renmin Hospital at Wuhan University. Patients who had at least two consecutive spontaneous abortions and were 7~10 weeks pregnant were allocated to the RSA group (n = 10) and the women with no history of spontaneous abortion and also with a gestation period of 7~10 weeks were enrolled in the control group (n = 10). All participants were non-smokers, not on medication, and had regular menstrual cycles. The ages of the women in the RSA group ranged from 19~33 years old with an average age of 25.6 ± 5.1 years. The gestational ages at the time of analysis were 6-10 weeks with an average of 9.0 ± 3.7 weeks. The ages of the women in the control group ranged from 20 to 35 years old with an average age of 24.3 ± 5.6 years, and the gestational ages at the time of analysis were 4.5 to 10 weeks with an average of 8.3 ± 3.9 weeks. The differences in age and gestational weeks between the two groups were not significant ($P > 0.05$). Gestational age was confirmed by both the last menstrual period and ultrasound. All samples were immediately fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemistry.

Immunohistochemical analysis

Trophoblastic tissues were obtained from dilation and curettage operations and then fixed and embedded in paraffin after washing and rinsing in normal saline. Afterwards, the tissues were incised into sections of 4 μm thickness and then dewaxed, rehydrated, and incubated with hydrogen peroxide and 1% bovine serum albumin/TBS. Thereafter, the tissue sections were incubated in mouse anti-human CD44v6 antibody (1:200; VFF-18, Bender MedSystems, Austria), anti-NF-κB p65 (1:1000; NF-12; Sigma, USA), or mouse/rabbit IgG of homologous type overnight at 4°C. After washing three times with TBS, the sections were blocked with peroxidase-conjugated goat anti-mouse/rabbit IgG (Golden Bridge International, Inc., Beijing, China) and then the reaction was developed with 3,3'-diaminobenzidine and counterstained

with hematoxylin. All of the results were evaluated by the same pathologist. The experiments were repeated three times.

siRNA inhibition

Cells were transfected with CD44v6-specific siRNA (RiboBio, Guangzhou, China): sense strand 5'-GAACAG UGGUUUGGCAACA dTdT-3', antisense strand 3'-dTdT CUUGUCACCAAACCGUUGU-5', using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol. Forty-eight hours later, quantitative RT-PCR and immunofluorescence were conducted on the transfected cells.

Immunofluorescence

Localization and comparison of the distribution of CD44v6 and NF-κB were confirmed by immunofluorescence analysis. HTR-8/SVneo cells were cultured on 24-well plates with poly-L-lysine-coated cover slips and then transfected with CD44v6 siRNA or control vectors. Forty-eight hours later, the transfected cells were washed three times with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde-PBS for 10 minutes. Finally, the cells were stained with the primary antibodies (1:200 dilution for the anti-CD44v6 antibody, 1:1000 dilution for anti-NF-κB) at 4°C for 10 hours and incubated with the secondary antibody (Boster Biological Technology, Ltd., China) conjugated with carbocyanine 3 (Cy3) or Alexa Fluor 488 for 2 hours at 37°C. Primary antibody alone was used as a negative control. Confocal laser scanning microscopy (CLSM) (TCS SP2, Germany) was used for evaluation of morphology determination.

Quantitative RT-PCR

To evaluate the gene silencing effect of the siRNA targeting CD44v6, reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time polymerase chain reaction (qRT-PCR) were performed in HTR-8/SVneo cells 48 hours after transfection with Lipofectamine. First, total RNA was extracted from cells using TRIzol Reagent (Invitrogen, CA, United States) and complementary DNA (cDNA) was synthesized using a PrimeScript RT kit (Takara, Dalian, China), following the manufacturer's guidance. Then, cDNA was used for qRT-PCR with the SYBR PrimeScript RT-PCR kit (Takara, Dalian, China). Primers for CD44V6 amplification were as follows: forward: 5'-GCCTTTGATGGACCAATTACC-3'; reverse: 5'-TCATTCCTATTGGTAGCAGGGA-3'. Primers for NF-κB amplification were as follows: forward: 5'-CGCATCCAGACCAACAACA-3'; reverse: 5'-TGCCAGAGTTTCGGTTCAC-3'. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control with forward primer: 5'-GGTCGGAGTCAACGGATTTG-3'; reverse primer: 5'-GGAAGATGGTGATGGGATTTC-3'. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method [23]. All experiments were performed in triplicate and the results were expressed as the mean of 3 independent measurements.

Colony formation assay

To evaluate the effect of CD44v6 on trophoblast proliferation, a colony formation assay was performed [24]. After transfection with CD44v6 siRNA or negative control siRNA for 3 days, the cell lines were seeded into six-well plates at 1×10^3 cells/well. After 14 days, the colonies were fixed with methanol, stained with crystal violet, and photographed. All experiments were repeated three times.

Transwell invasion assay

The invasive ability of trophoblasts was evaluated by the Transwell Matrigel invasion assay. Cell culture inserts of 8-mm pore size and 6.5-mm diameter (Corning Inc., NY) were coated with 25 mL of Matrigel and placed in 24-well plates. HTR-8/SVneo cells were cultured for 48 hours after transfection with CD44v6 siRNA or negative control siRNA and then 1×10^5 cells in 200 mL of DMEM/F12 were placed in the upper chamber of each insert. The lower chambers were filled with 800 mL of DMEM/F12 containing 10% fetal bovine serum and the plates were incubated at 37°C for 48 hours. Thereafter, the inserts were removed for washing in ice-cold PBS and by wiping with a cotton bud, noninvading cells were removed from the upper surface of the filter. Cells on the lower surface of the inserts were fixed in 4% paraformaldehyde, stained with crystal violet, and observed using an inverted phase-contrast microscope (Leica). Finally, cells that had invaded to the lower surface were counted at a magnification of 200 ×. The results were evaluated by two independent researchers and the invasion index was calcu-

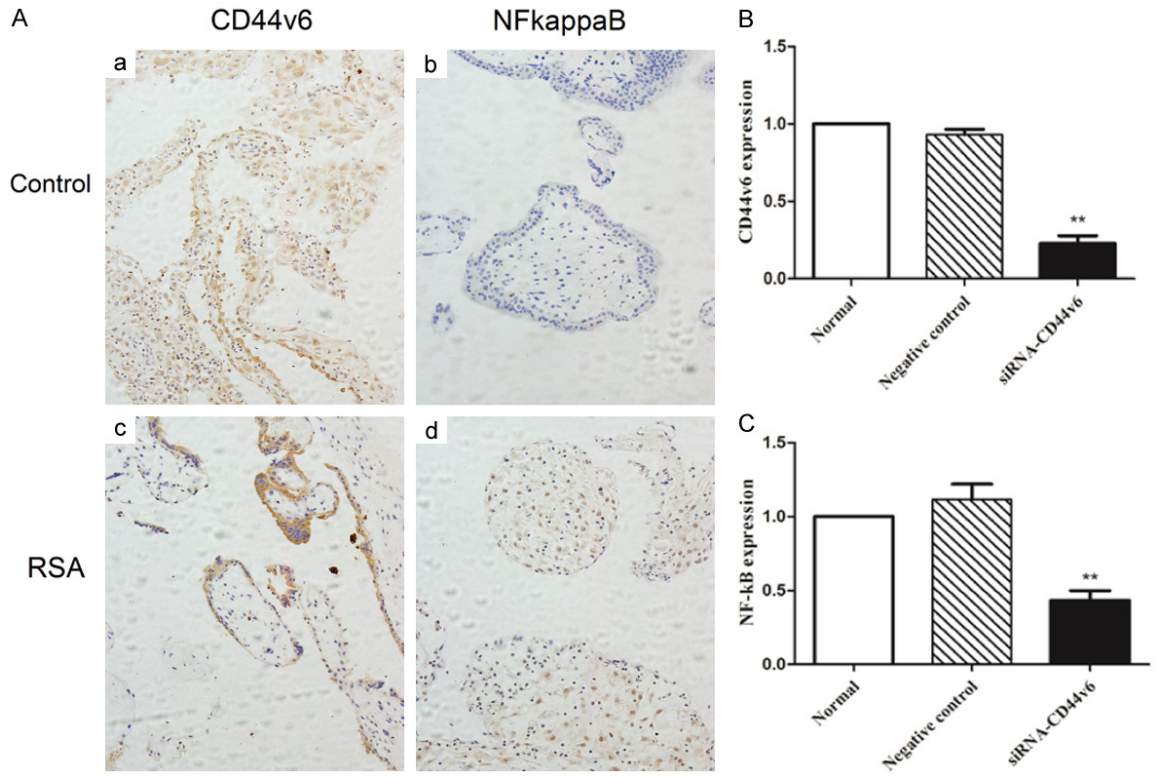

Figure 1. CD44v6 expression is decreased in villi from RSA patients compared to those from normal pregnant women and inhibition of CD44v6 with CD44v6-specifc siRNA suppresses expression of NF-κB in HTR-8/SVneo cells. A. Representative images of CD44v6 expression in villous tissue. Immunostaining verifies CD44v6 expression in tissue sections, a brownish color represents positive staining, magnification: 200 ×; B and C. Real-time PCR analysis of CD44v6 and NF-κB mRNA in HTR-8/SVneo cells infected with CD44v6 siRNA or control siRNA (**P < 0.01).

lated as the proportion of the invading cells in each experimental group expressed relative to control cells. Each process was repeated three times independently.

Statistical analysis

Data were expressed as mean ± standard deviation. One-way analysis of variance was applied for the measurement of differences among groups. Differences were considered statistically significant when $P < 0.05$. Data were analyzed using SPSS software (v.13.0).

Results

CD44v6 and NF-κB expression was lower in patients with RSA than in those with normal pregnancy

We conducted immunohistochemistry in villi isolated from patients with either threatened abortion or healthy pregnancy to analyze the expression of CD44v6 and NF-κB. The results showed that CD44v6 and NF-κB were expressed in the villous trophoblasts during the first trimester. In addition, both CD44v6 and NF-κB expression levels were significantly decreased in the threatened abortion patients compared to the women with normal pregnancies (**Figure 1A**).

Knockdown of CD44v6 suppresses expression of NF-κB and the proliferation and migration of trophoblast cells

After transfection with CD44v6 siRNA, mRNA expression level of CD44v6 in HTR-8/SVneo cells was verified by RT-PCR (**Figure 1B**). The results demonstrated that expression of CD44v6 was significantly decreased in transfected group compared to the control group. To investigate the effect of CD44v6 on the proliferating and migrating functions of trophoblast cells, both colony and Transwell invasion assays were conducted 48 hours after transfection. We found that both proliferation and migration of CD44v6 siRNA-transfected cells were signifi-

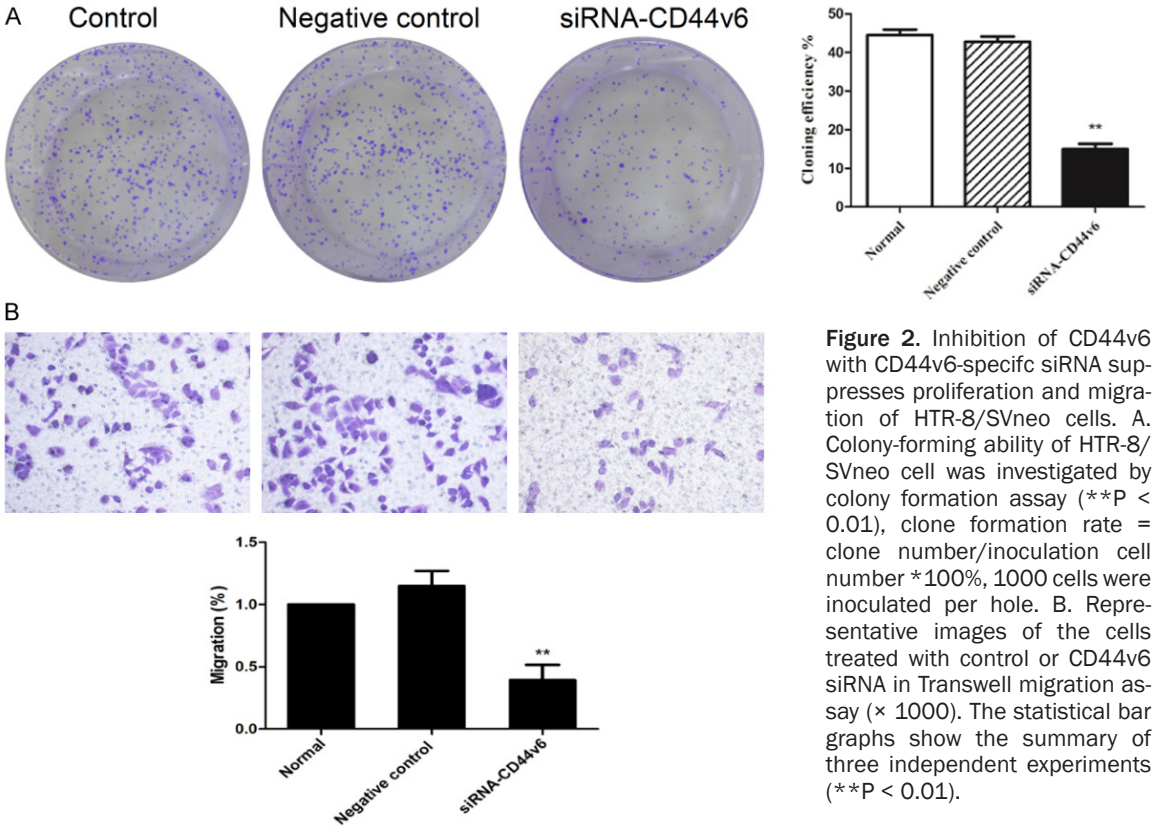

Figure 2. Inhibition of CD44v6 with CD44v6-specifc siRNA suppresses proliferation and migration of HTR-8/SVneo cells. A. Colony-forming ability of HTR-8/SVneo cell was investigated by colony formation assay (**P < 0.01), clone formation rate = clone number/inoculation cell number *100%, 1000 cells were inoculated per hole. B. Representative images of the cells treated with control or CD44v6 siRNA in Transwell migration assay (× 1000). The statistical bar graphs show the summary of three independent experiments (**P < 0.01).

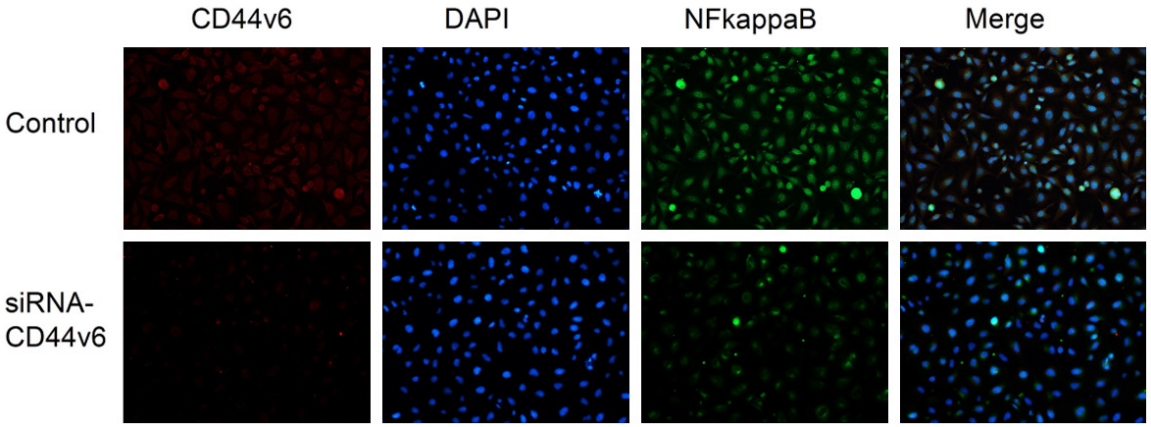

Figure 3. Knockdown of CD44v6 attenuates nuclear translocation of NF-κB in HTR-8/SVneo cells. Fluorescent signals specific to CD44v6 antibody were visualized as red, NF-κB (green), and the nuclei staining with DAPI (blue).

cantly decreased compared with those of control cells treated with vehicle (**Figure 2**).

To interpret the mechanism by which CD44v6 influences the function of trophoblasts, we also measured expression of NF-κB by RT-PCR in trophoblast cells transfected with CD44v6 siRNA or not and we found that expression of NF-κB was significantly decreased in CD44v6-knockdown HTR-8/SVneo cells (**Figure 1C**).

Knockdown of CD44v6 attenuates nuclear translocation of NF-κB in trophoblast cells

To gain further insight into the molecular events underlying the ability of CD44v6 to regulate trophoblast function, HTR-8/SVneo cells were transfected with CD44v6 siRNA or control vehicle for 48 hours. Confocal imaging showed that NF-κB was distributed mainly in the nuclei of the cells whereas knockdown of CD44v6 obvi-

ously reduced the nuclear accumulation of NF-κB (**Figure 3**).

Discussion

During pregnancy, trophoblast cell proliferation, migration, and invasion are crucial for the development of a normal placenta and fetus. The entire process must be strictly controlled [25]. Any abnormal regulation may lead to pregnancy failure and even recurrent spontaneous abortion.

CD44 is a type 1 transmembrane glycoprotein that mainly participates in cell-cell and cell-matrix interactions by acting as a cell surface receptor of the adhesion molecules. It is expressed as the standard form (CD44s) or as one of 12 variant isoforms (CD44-v1 to CD44-v12) [26, 27]. The variant CD44v6 is the main isoform reported to be implicated in tumor cell invasion and metastasis in various types of cancers such as colon cancer, pancreatic carcinoma, laryngeal carcinoma, gastric cancer, breast cancer, and other cancers [28-32]. To some extent, trophoblast cells share some common characteristics with tumor cells such as proliferative and migratory potential, suggesting that CD44v6 may play a role in trophoblast function.

It has long been confirmed that CD44v6 is expressed in the placenta [13] and immunohistochemistry experiments have shown that expression of CD44v6 decreased over time from first-trimester villi to term placenta, indicating that CD44v6 might play an important role in trophoblast cell invasion into maternal tissue during early pregnancy [33]. Furthermore, when female rats received anti-CD44v6 starting at the time of mating, conception was infrequent, even when the potential mating period was extended up to 4 days [14]. However, the exact function and relevant mechanisms of CD44v6 related to trophoblast function and pregnancy outcomes have yet to be determined.

The NF-κB family includes five members: p65 (RelA), RelB, c-Rel, p105/p50, and p100/p52. All members possess a conserved Rel homology domain in the N-terminal region which is responsible for the central functions of these proteins such as homo- and heterodimerization, nuclear localization, and DNA binding [18]. In the static state, p65-p50 dimers are bound to inhibitor κB proteins (IκBs) which maintains NF-κB in steady state in the cytosol [34-37]. Upon activation, IκBα is phosphorylated by its inhibitor κB kinase (IKK) complex, followed by ubiquitination and proteasomal degradation. Then, NF-κB detaches from IκBα and accumulates in the nucleus to carry out its DNA binding, target gene transcription promotion, and other functions.

Rachid Marhaba reported that when mouse thymoma EL4 cells were transfected with CD44v6 cDNA, the expression levels of JNK, c-Jun, and IκBa were significantly increased and NF-κB was partially translocated into the nucleus [38]. There has also been a study demonstrating that in primary human melanocytes, CD44v6 could interact with the NFκB/Egr-1/C/EBP-beta complex and regulate the mobility of melanocytes [39]. Recently, Misra MK used a different model to predict idiopathic recurrent miscarriage (IRM) risk and the results showed that two functional variants of NFκB1 individually increased the risk by two- to nearly three-fold and together increased the risk by 10.5-fold, suggesting the association of NFκB1 gene variants with IRM risk [21]. Moreover, Esencay revealed that NF-κB may prevent induction of programmed cell death and enhance trophoblast proliferation [40]. Wang's group [41] confirmed that ERK/NF-κB signaling promotes trophoblast nuclear antigen expression and consequently induces trophoblast proliferation. These phenomena indicate the possible involvement of the CD44v6-NFκB signaling pathway in RSA.

In this regard, we first confirmed that expression of CD44v6 and NFκB was decreased in the villi of RSA patients compared with the villi of women with healthy pregnancies. Then, we knocked down CD44v6 expression by specific CD44v6 siRNA and qRT-PCR analysis showed that NFκB expression was downregulated. Meanwhile, proliferation and migration of trophoblast cells were significantly suppressed. Considering that the activated form of NFκB was mainly located in the nuclei, we further conducted immunofluorescence experiments to assess the subcellular localization of CD44v6 and NF-κB. These experiments indicate that when CD44v6 is knocked down by specific siRNA in the trophoblast cell line HTR-8/SVneo,

the nuclear translocation of NF-κB is significantly attenuated. Thus, it is likely that insufficient expression of CD44v6 reduces the proliferative and migratory potential of trophoblast cells by downregulating expression of NF-κB and its translocation to the nucleus, eventually resulting in RSA.

Our study suggests possible mechanisms related to the regulation of trophoblast function, which eventually contributes to development of RSA. Further research should be conducted on the target genes, functional molecules, and proteins with which NFκB interacts to help further elucidate the mechanisms of RSA.

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Disclosure of conflict of interest

None.

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