Overexpression of long non-coding RNA NEAT1 enhances cell viability and inhibits apoptosis in recurrent spontaneous abortion by targeting the miR-125b/BCL-2 axis

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Abstract. The current study aimed to investigate the function of the long non-coding RNA nuclear paraspeckle assembly transcript 1 (NEAT1) in the pathogenesis of recurrent spontaneous abortion (RSA) and to examine its potential mechanism. The expression of NEAT1, microRNA (miR)-125b and Bcl-2 in the villi of patients with RSAs and women with normal pregnancies was measured by reverse transcription-quantitative PCR. Cell viability was detected by the MTT assay and cell apoptosis was evaluated by flow cytometry. A dual-luciferase reporter assay was performed to verify the associations between NEAT1 and miR-125b. The protein expression of Bcl-2 was detected by western blot analysis. In the present study, the expression of NEAT1 and Bcl-2 was reduced and that of miR-125b was increased in clinical samples of villus tissues from patients with RSAs. *In vitro*, overexpression of NEAT1 enhanced the viability and suppressed the apoptosis of JEG-3 cells. It was demonstrated that miR-125b acts as a molecular sponge of NEAT1 and its expression was negatively regulated by NEAT1. miR-125b overexpression reduced the viability and promoted the apoptosis of JEG-3 cells. The expression of BCL-2, a target gene of miR-125b, was inversely correlated with that of miR-125b. Overexpression of miR-125b and inhibition of BCL-2 partially reversed the effect of NEAT1 overexpression on the viability and apoptosis of JEG-3 cells. Collectively, it was demonstrated that the NEAT1/miR-125b/BCL-2 axis plays a pivotal role in regulating the viability and apoptosis of JEG-3 cells. The findings of the present study offer new insights into the pathogenesis of RSA and may provide information on RSA treatment.

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Introduction

Recurrent spontaneous abortion (RSA), also known as recurrent pregnancy loss or recurrent miscarriage, is a complication of pregnancy affecting 1-2% of fertile couples (1). It refers to the occurrence of three or more consecutive spontaneous abortions and causes severe physical and mental harm to affected patients (2). Chromosomal abnormalities, pathogenic infections, immune disorders and genetic mutations are factors that contribute to RSA (3,4). Nevertheless, for ~50% of patients with RSAs, the causative factors remain unknown (5). Hence, it is important to investigate the underlying pathogenesis of RSA and identify available targets for its therapy.

Long non-coding RNAs (lncRNAs) are a group of non-coding RNAs with a length >200 nt (6). Previous studies have reported that lncRNAs affect the progression of RSA at the cellular level. For example, downregulation of the lncRNA metastasis associated lung adenocarcinoma transcript 1 inhibited the cell proliferation and migration, and increased apoptosis of human umbilical vein endothelial cells (7). Overexpression of HOX antisense intergenic RNA promoted trophoblast cell invasion and migration (8). Notably, the lncRNA nuclear paraspeckle assembly transcript 1 (NEAT1) was reported to affect cell proliferation and migration in several human cancer types such as endometrial (9), breast (10) and cervical cancer (11). Recently, Wang *et al* (12) discovered that NEAT1 expression was significantly reduced in the villi of patients experiencing recurrent miscarriages. However, the underlying function of NEAT1 in the pathogenesis of RSA has rarely been investigated.

MicroRNAs (miRNAs/miRs) are a type of conserved non-protein coding RNA (~22 nt) that participate in biological processes including cell differentiation, growth, apoptosis and angiogenesis (13,14). Previous studies have revealed that abnormal expression of miRNAs was associated with increased risk of RSA. For instance, upregulation of miR-27a (15) and miR-34a (16) and downregulation of miR-146a-5p (17) may contribute to RSA. Additionally, elevated miR-125b expression has been observed in decidual and villi tissues of patients with RSAs and has been suggested to be associated with RSA development (16,18). The interactions between lncRNAs and miRNAs, such as lncRNA SNHG7-1/miR-34a (19) and lncRNA H19/miR-106a-5p (20), reportedly affect the development of RSA. Nevertheless, the detailed regulatory mechanisms of

miR-125b and its interaction with NEAT1 in the progression of RSA remain unclear.

Herein, the expression levels of NEAT1, miR-125b and BCL-2 in the villus tissues of patients with RSAs was evaluated and the regulatory mechanism of the NEAT1/miR-125b/BCL-2 axis on RSA pathogenesis was investigated *in vitro*. The present study aimed to elucidate the molecular mechanism underlying RSA and reveal possible targets for RSA treatment.

Materials and methods

Patients and clinical samples. In this retrospective study, villus tissue samples were obtained from patients with RSAs and healthy controls who visited Liaocheng Dongchangfu District Maternal and Child Health Hospital (Liaocheng, China) between March 2018 and February 2019. The RSA group included 20 Chinese women (age range, 25-35 years old; mean age, 29.84±3.44 years old) with a history of three or more spontaneous abortions. The control group included 20 age-matched women (age range, 24-35 years old; mean age, 29.23±3.01 years old) with the request for termination of pregnancy because of unplanned pregnancy. The inclusion criteria for both groups were as follows: i) No chromosomal abnormalities; ii) normal reproductive endocrinology; iii) no diabetes, thyroid dysfunction or other systemic diseases; and iv) no organic deformity of the uterus and genital tract. The villus samples were obtained from cases of induced abortion at 5-10 gestational weeks. All samples were cleaned in sterile saline to remove excess blood, mucus and deciduas, and stored in liquid nitrogen cans. The present study was approved by the Ethics Committee of Liaocheng Dongchangfu Maternal and Child Health Hospital and all participants signed informed consent.

Cell culture and transfection. The human placental choriocarcinoma cell line (JEG-3) was purchased from the Cell Bank of Type Culture Collection (Chinese Academy of Sciences). JEG-3 cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in an incubator containing 5% CO_2.

Short hairpin RNA NEAT1 (sh-NEAT1) and its negative control (sh-NC), sh-BCL-2, miR-125b mimics (5'-UCCCUGAGACCCUAACUUGUGA-3') and their corresponding negative control (mimics NC, 5'-UUCUCCGAACGUGUCACGUTT-3'), miR-125b inhibitors (5'-UCACAAGUUAGGGUCUCAGGGA-3') and their corresponding negative control (inhibitor NC, 5'-CAGUACUUUUGUGUAGUACAA-3') and pcDNA-NEAT1 and its corresponding negative control (pcDNA-NC) were purchased from Shanghai GenePharma Co., Ltd. JEG-3 cells were seeded into 6-well plates and allowed to grow until the confluence reached 80%. The cells were then transfected with the previously mentioned vectors (all at 20 nM) using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C. Subsequently, 48 h after transfection, cells were harvested to perform further experiments.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from villus tissues and JEG-3 cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. A volume of 2 μl RNA (1 μg/μl) was reverse-transcribed into cDNA at 42˚C for 45 min using a First-Strand cDNA Synthesis kit (Takara Biotechnology Co., Ltd.), and qPCR was carried out using the SYBR Green PCR Kit (Takara Biotechnology Co., Ltd.). The thermocycling conditions for qPCR were as follows: Initial denaturation at 94˚C for 5 min; 40 cycles of 94˚C for 15 sec; 60˚C for 30 sec and 72˚C for 1 min. The expression of NEAT1 and Bcl-2 was normalized to that of GAPDH, and U6 served as the internal control for miR-125b. The data were analyzed using the $2^{-\Delta\Delta Cq}$ method (21). The primer sequences are listed in Table I.

MTT assay. Transfected JEG-3 cells were seeded into 96-well plates at $3x10^3$ cells/well and incubated with 20 μl of 5 mg/ml MTT reagent (Sigma-Aldrich; Merck KGaA) for 4 h at 37˚C. Subsequently, 200 μl of dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) was added to each well for 10 min at 37˚C to dissolve the formazan crystals. The absorbance of the wells was detected using a microplate reader at 540 nm.

Flow cytometry. Cell apoptosis assay was measured using an Annexin V Apoptosis Detection kit (cat. no. BMS500FI-300; Thermo Fisher Scientific, Inc.). Following 24 h of transfection, JEG-3 cells were washed with ice cold PBS and centrifuged (450 x g for 20 min at 4˚C). The cells were then resuspended in 1x binding buffer and incubated with annexin V-FITC and PI (Thermo Fisher Scientific, Inc.) at 25˚C in the dark for 20 min. Apoptotic cell populations were assessed using a FACScan™ flow cytometer (BD Biosciences) and the data were analyzed using FlowJo software (version 0.9.18, FlowJo LLC).

Dual-luciferase reporter assay (DLR). The targeting relationships between miR-125b and NEAT1 or BCL-2 were analyzed using StarBase database (version 2.0; http://starbase.sysu.edu.cn). The predicted wild-type (WT) or mutant (MUT) 3'-untranslated regions of NEAT1/BCL-2 containing miR-125b binding sites were inserted into pGL3-basic vectors (Promega Corporation) to produce NEAT1 WT, NEAT1 MUT, BCL-2 WT and BCL-2 MUT plasmids. JEG-3 cells (2,000 cells/well) were cultured in 24-well plates until the confluence reached 80% and transfected with WT or MUT luciferase reporters and miR-125b mimics or miR-NC mixed with Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. After 24 h at 37˚C, luciferase activity was detected by running a Dual-Luciferase Assay system (Promega Corporation). *Renilla* luciferase served as an endogenous control.

Western blot analysis. JEG-3 cells were lysed in RIPA buffer (Beyotime Institute of Biotechnology) to obtain total protein. The protein concentration was detected by the BCA Protein Assay Kit (Abcam). A total of 50 μg of protein/lane was separated by 10% SDS-PAGE, and then transferred to PVDF membranes and immersed in 5% skimmed milk for 1 h at 25˚C to block non-specific binding. The membranes were subsequently incubated overnight at 4˚C with primary antibodies against BCL-2 (1:1,000; cat. no. ab32124; Abcam), β-actin (1:1,000, ab5694, Abcam), pro caspase 3 (1:1,000; cat. no. ab32150; Abcam) and cleaved caspase 3 (1:1,000;

Table I Primers for RT-qPCR.

Gene	Forward (5'→3')	Reverse (5'→3')
NEAT1	CTTCCTCCCTTTAACTTATCCATTCAC	CTCTTCCTCCACCATTACCAACAATAC
MiR-125b	GTCCCTGAGACCCTAACTTG	AGCCTAACCCGTGGATTT
BCL-2	CTGCACCTGACGCCCTTCACC	CACATGACCCCACCGAACTCAAAGA
GAPDH	GCGAGATCGCACTCATCATCT	TCAGTGGTGGACCTGACC
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

NEAT1, nuclear paraspeckle assembly transcript 1; miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR.

cat. no. ab32042; Abcam). Following the primary incubation, membranes were incubated with secondary antibody HRP-conjugated anti-rabbit IgG (1:5,000; cat. no. ab205718; Abcam) at 37˚C for 1 h. Protein signals were detected using the ECL Plus reagent (Beyotime Institute of Biotechnology) and the immunoblots were quantified using ImageJ software (version 4.0; Bio-Rad Laboratories, Inc.).

Statistical analysis. All statistical analyses were conducted using SPSS 22.0 software (IBM Corp.). The data are presented as the mean ± standard deviation. Differences between two groups were compared using unpaired Student's t-test and those between multiple groups were compared using one-way analysis of variance followed by Tukey's post hoc test. Variable correlation was evaluated through Pearson's correlation analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

NEAT1 overexpression enhances viability and inhibits apoptosis of JEG-3 cells. In order to examine the role of NEAT1 in the pathogenesis of RSA, NEAT1 expression was detected in the villi of patients with RSAs and women with normal pregnancies. The mRNA expression of NEAT1 in the villi of patients with RSAs was found to be significantly lower than that in the villi of women with normal pregnancies (Fig. 1A). After pcDNA-NEAT1 transfection, NEAT1 mRNA expression was enhanced in JEG-3 cells compared with that in cells transfected with pcDNA-NC, while NEAT1 knockdown caused the opposite effect (Fig. 1B). The MTT assay demonstrated that the cell viability was significantly increased in the pcDNA-NEAT1 group compared with the pcDNA-NC group; while compared to the sh-NC group, cell viability in the sh-NEAT1 was reduced (Fig. 1C). Meanwhile, apoptosis rate was significantly inhibited in the pcDNA-NEAT1 group compared with the pcDNA-NC group, while it was promoted in the sh-NEAT1 group compared with the sh-NC group (Fig. 1D). To validate the observation on apoptosis, western blot analysis was performed to evaluate the expression of cleaved caspase 3/pro-caspase-3 ratio. This ratio was significantly suppressed in JEG-3 cells transfected with pcDNA-NEAT1 compared with those transfected with pcDNA-NC, whereas it was significantly elevated after sh-NEAT1 transfection compared with sh-NC transfection (Fig. 1E). These data demonstrate that NEAT1 overexpression enhances the viability and inhibits the apoptosis of JEG-3 cells.

miR-125b acts as a target of NEAT1. Possible miRNA targets of NEAT1 were studied in order to understand the regulatory mechanism of NEAT1 in RSA development. The present study demonstrated that NEAT1 contained complementary binding sites to miR-125b (Fig. 2A). DLR assay demonstrated that the luciferase activity in the miR-125b mimics/NEAT1 WT group was significantly reduced compared with the mimics NC/NEAT1 WT group (Fig. 2B). In addition, miR-125b mRNA expression was expressed at significantly higher levels in the villi of patients with RSAs compared to those in the villi of women with normal pregnancies (Fig. 2C). Furthermore, the mRNA expression of miR-125b was negatively correlated with that of NEAT1 (Fig. 2D), as NEAT1 mRNA overexpression suppressed miR-125b mRNA expression in JEG-3 cells but NEAT1 silencing upregulated miR-125b, compared with their respective negative controls (Fig. 2E). These results reveal that NEAT1 directly targets and negatively modulates the expression of miR-125b.

miR-125b overexpression suppresses viability and promotes apoptosis of JEG-3 cells. To examine the effect of miR-125b in RSA progression, JEG-3 cells were transfected with miR-125b mimics or inhibitors, which significantly enhanced and reduced the mRNA expression levels of miR-125b, respectively, compared with their respective negative controls (Fig. 3A). The present study demonstrated that miR-125b overexpression suppressed the viability and promoted the apoptosis of JEG-3 cells compared with mimics NC; whereas, compared with inhibitor NC, miR-125b inhibition induced the opposite effect (Fig. 3B and C). Furthermore, western blot analysis demonstrated that cleaved caspase 3/pro-caspase-3 ratio was upregulated by miR-125b overexpression compared with mimics NC and downregulated by miR-125b inhibition compared with inhibitor NC (Fig. 3D).

MiR-125b directly targets BCL-2. The potential target site between BCL-2 and miR-125b was predicted using Starbase (Fig. 4A). DLR assay revealed that the luciferase activity in the miR-125b mimics/BCL-2 WT group was significantly reduced compared with the mimics NC/BCL-2 WT group (Fig. 4B) in JEG-3 cells. Moreover, the mRNA expression of BCL-2 in the villi of patients with RSAs as markedly lower than that in the villi of normal pregnancies (Fig. 4C)

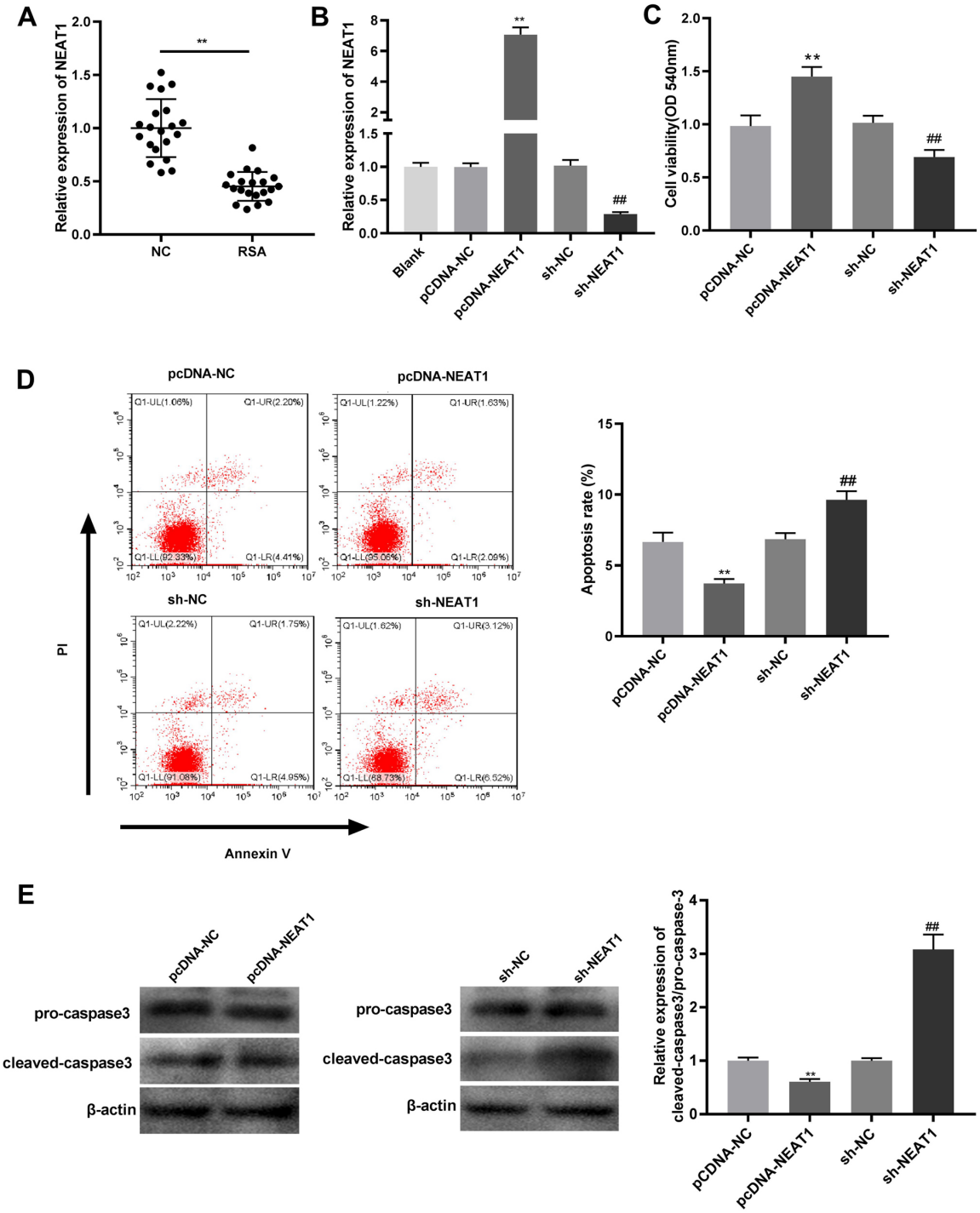

Figure 1. NEAT1 overexpression promotes cell viability and inhibits apoptosis in JEG-3 cells. (A) NEAT1 expression was detected by RT-qPCR in villi of RSA patients and normal pregnancy villi. **P<0.01 vs. normal pregnancy villi. (B) NEAT1 expression was detected by RT-qPCR in JEG-3 cells transfected with pcDNA-NC, pcDNA-NEAT1, sh-NC or sh-NEAT1. **P<0.01 vs. pcDNA-NC; $^{\#\#}$P<0.01 vs. sh-NC. (C) Cell viability was detected by MTT assay in JEG-3 cells transfected with pcDNA-NC, pcDNA-NEAT1, sh-NC or sh-NEAT1. **P<0.01 vs. pcDNA-NC; $^{\#\#}$P<0.01 vs. sh-NC. (D) Flow cytometry was used to detect the apoptotic rates of JEG-3 cells transfected with pcDNA-NC, pcDNA-NEAT1, sh-NC or sh-NEAT1. **P<0.01 vs. pcDNA-NC; $^{\#\#}$P<0.01 vs. sh-NC. (E) Ratio of protein expression of cleaved caspase3/pro-caspase was detected by western blot analysis in JEG-3 cells transfected with pcDNA-NC, pcDNA-NEAT1, sh-NC or sh-NEAT1. **P<0.01 vs. pcDNA-NC; $^{\#\#}$P<0.01 vs. sh-NC. NEAT1, nuclear paraspeckle assembly transcript 1; RT-qPCR, reverse transcription-quantitative PCR; RSA, recurrent spontaneous abortion; NC, negative control; sh, short hairpin.

and BCL-2 mRNA expression was negatively correlated with that of miR-125b (Fig. 4D). MiR-125b overexpression significantly downregulated BCL-2 compared with mimics NC; whereas, compared with inhibitor NC, miR-125b inhibition significantly enhanced BCL-2 protein expression in JEG-3 cells (Fig. 4E). These data confirm that miR-125b directly targets BCL-2 and negatively regulates BCL-2 expression.

NEAT1 overexpression enhances cell viability and inhibits apoptosis by regulating the miR-125b/BCL-2 axis. The transfection efficiency of sh-BCL-2 was detected by RT-qPCR.

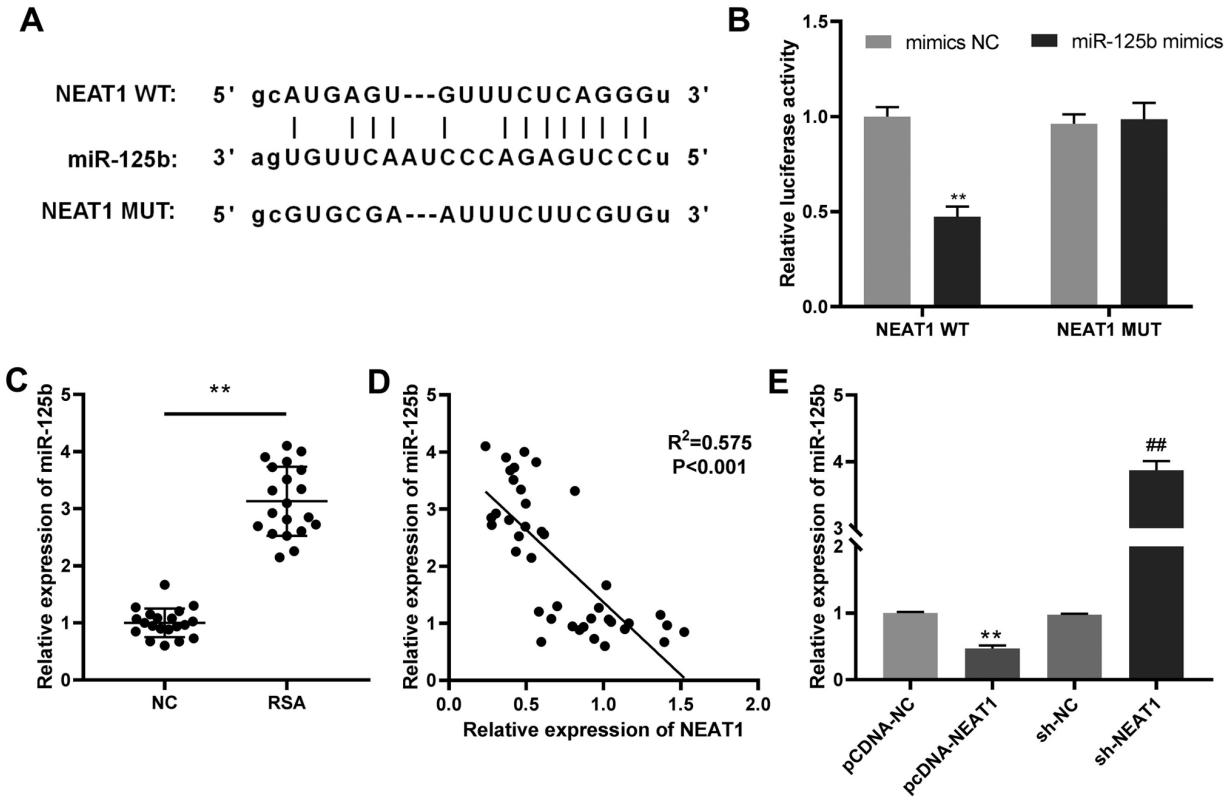

Figure 2. miR-125b acts as a target of NEAT1. (A) The target sites between NEAT1 and miR-125b were predicted by Starbase. (B) Dual-luciferase assay confirmed the association between NEAT1 and miR-125b in JEG-3 cells. **P<0.01 vs. mimics NC. (C) The expression of miR-125b was measured by RT-qPCR in villi of patients with RSAs and normal pregnancy. **P<0.01 vs. villi from patients with normal pregnancy. (D) The correlation between NEAT1 and miR-125b was evaluated by Pearson's correlation analysis. R^2=0.575, P<0.001. (E) The expression of miR-125b was measured by RT-qPCR in JEG-3 cells transfected with pcDNA-NC, pcDNA-NEAT1, sh-NC or sh-NEAT1. **P<0.01 vs. pcDNA-NC; ##P<0.01 vs. sh-NC. miR, microRNA; NEAT1, nuclear paraspeckle assembly transcript; NC, negative control; RT-qPCR, reverse transcription-quantitative PCR; RSA, recurrent spontaneous abortion; sh, short hairpin.

The mRNA expression of BCL-2 in JEG-3 cells transfected with sh-BCL-2 was significantly decreased compared with sh-NC (Fig. 5A), suggesting that sh-BCL-2 was transfected successfully. Rescue experiments were subsequently conducted to examine the association between NEAT1 and the miR-125b/BCL-2 axis in JEG-3 cells. NEAT1 mRNA overexpression significantly increased the protein expression of BCL-2 compared with pcDNA-NC, but this effect was inhibited by miR-125b overexpression and BCL-2 knockdown in JEG-3 cells (Fig. 5B). Compared with pcDNA-NEAT1, overexpression of miR-125b and silencing of BCL-2 attenuated both the NEAT1-induced increase in viability and decrease in apoptosis in JEG-3 cells (Fig. 5C and D). Meanwhile, the results of the western blot analysis demonstrated that both miR-125b overexpression and BCL-2 suppression reversed the inhibitory effect of NEAT1 overexpression on the protein expression of cleaved caspase 3 (Fig. 5E). Overall, these findings suggest that NEAT1 overexpression enhances the viability and inhibits the apoptosis of JEG-3 cells by regulating the miR-125b/BCL-2 axis.

Discussion

RSA is a common complication during pregnancy which has a complex etiology (5). Normal trophoblast cells play a critical role in fetal growth, but failure in trophoblast transformation or impaired trophoblast function can lead to RSA (22). In the present study, NEAT1 (an RSA-associated lncRNA) was identified as a potential target for RSA therapy. The present study demonstrated that NEAT1 expression was decreased in the villi of patients with RSAs and NEAT1 overexpression enhanced the viability and inhibited the apoptosis of JEG-3 cells by interacting with the miR-125b/BCL-2 axis.

Numerous lncRNAs such as MALAT1 (7), HOTAIR (8) and TCL6 (23) have been reported to be abnormally expressed in RSA. In the present study, NEAT1 expression was downregulated in the villi of RSA patients. Similarly, a recent study demonstrated that NEAT1 expression was reduced in the villi of patients experiencing recurrent miscarriages (12). These data therefore suggest that altered NEAT1 expression may be associated with RSA pathogenesis. In addition, NEAT1 is a critical lncRNA that regulates cell processes, such as apoptosis and proliferation in human cancers (24-26). Wang *et al* (25) reported that NEAT1 overexpression increased the viability of endometrial cancer cells and Yuan *et al* (26) revealed that NEAT1 knockdown decreased the viability and enhanced the apoptosis of cervical cancer cells. Herein, it was demonstrated that NEAT1 overexpression enhanced the viability and suppressed the apoptosis of JEG-3 cells. In functional studies, there are some similarities between trophoblasts and cancer cells that may be related to their migration and invasion capabilities (27). The results of the present study corroborate those in previous studies suggesting that NEAT1 overexpression may protect patients

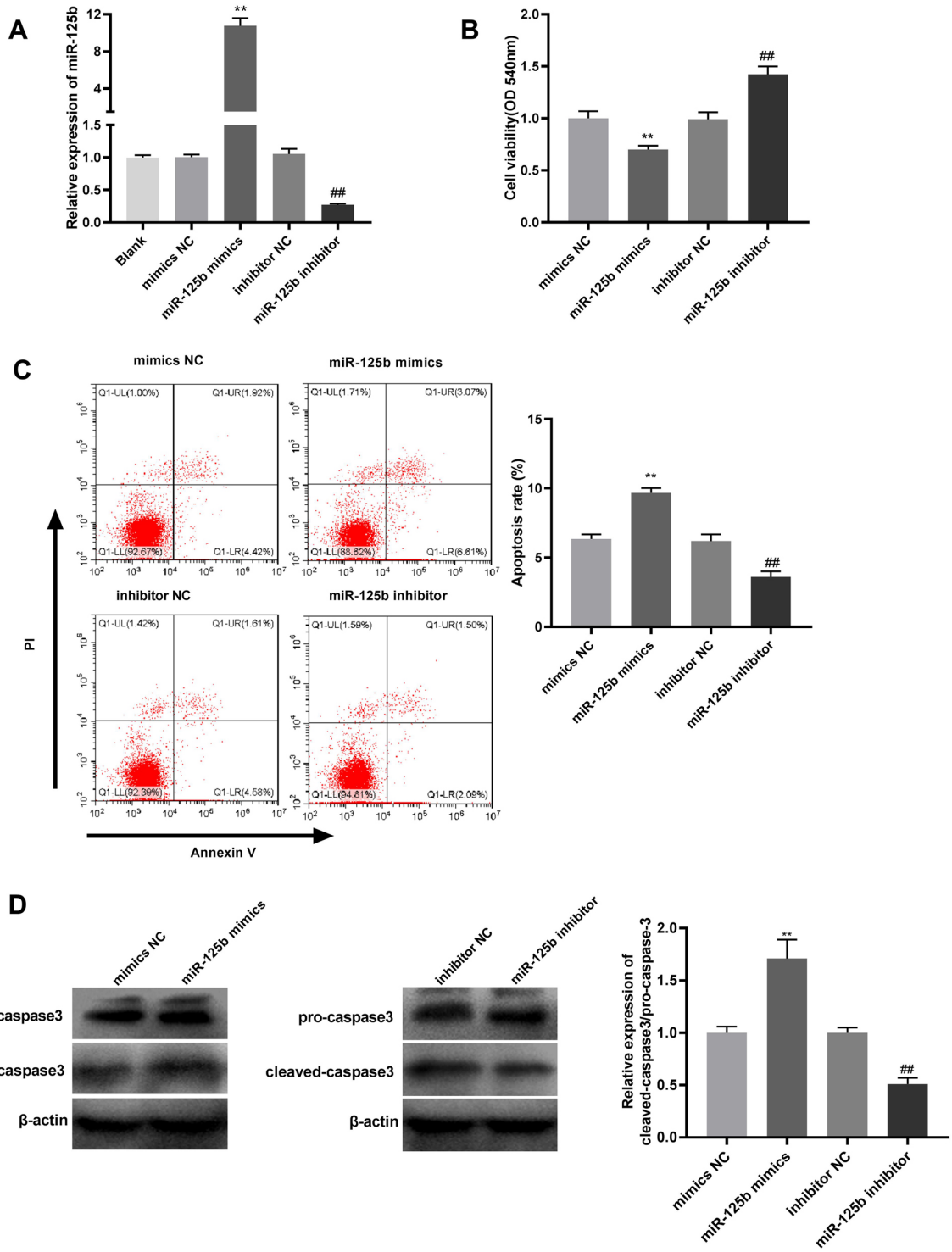

Figure 3. miR-125b overexpression inhibits cell viability and promotes apoptosis in JEG-3 cells. JEG-3 cells were transfected with mimics NC, miR-125b mimics, inhibitor NC, or miR-125b inhibitor. **P<0.01 vs. mimics NC; $^{\#\#}$P<0.01 vs. inhibitor NC. (A) The expression of miR-125b was measured by RT-qPCR. (B) Cell viability was detected by MTT assay. (C) Flow cytometry was used to detect the apoptotic rates. (D) The protein expression of cleaved caspase3 was detected by western blot analysis. miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR; NC, negative control.

against RSA by enhancing cell viability and inhibiting apoptosis.

Increasing evidence has demonstrated that miR-125b expression is increased in decidual (16) and villus tissues (18) of patients with RSAs. In the current study, the level of miR-125b was also discovered to be upregulated in the villi of patients with RSAs, suggesting that dysregulation of miR-125b expression may be associated with increased risk of RSA. Meanwhile, accumulating data have implicated the significant role of miR-125b in regulating cellular processes. For instance, miR-125b overexpression induced the apoptosis of trophoblast cells (28). In another study, increased miR-125b expression reduced cell viability and impaired the invasion and migration capacities of extra-villous trophoblastic cells (29). In the

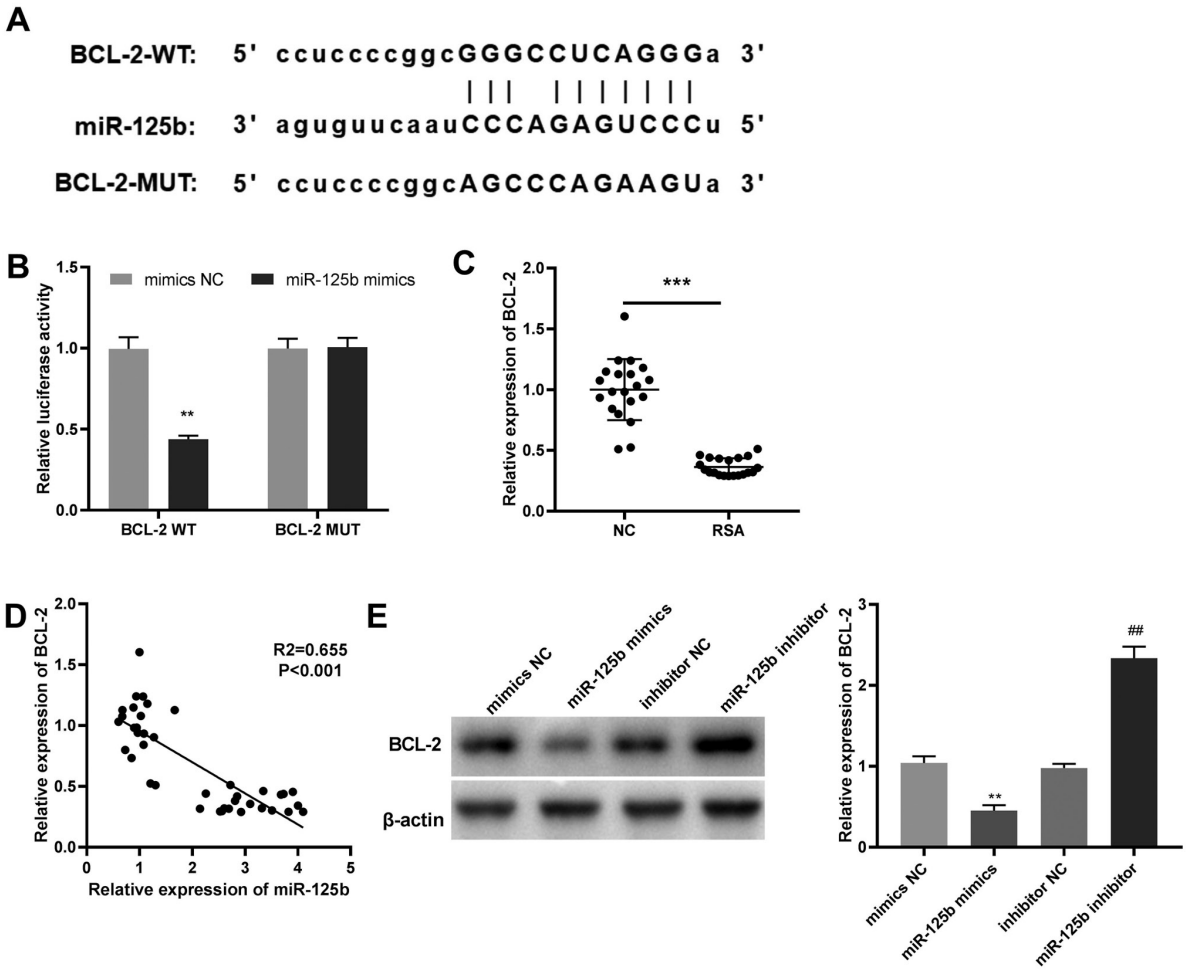

Figure 4. BCL-2 is a direct target of miR-125b. (A) The target sites between miR-125b and BCL-2 were predicted by Starbase. (B) Dual-luciferase assay confirmed the association between miR-125b and BCL-2 in JEG-3 cells. **P<0.01 vs. mimics NC. (C) The expression of BCL-2 was measured by RT-qPCR in villi of patients with RSAs and normal pregnancy. ***P<0.001 vs. normal pregnancy villi. (D) The correlation between miR-125b and BCL-2 was evaluated by Pearson's correlation analysis. R^2=0.655, P<0.001. (E) The protein expression of BCL-2 was detected by western blot in JEG-3 cells transfected with mimics NC, miR-125b mimics, inhibitor NC or miR-125b inhibitor. **P<0.01 vs. mimics NC; ##P<0.01 vs. inhibitor NC. miR, microRNA; NC, negative control; RT-qPCR, reverse transcription-quantitative PCR; RSA, recurrent spontaneous abortion; WT, wild type; MUT, mutant.

current study, miR-125b overexpression reduced the viability and promoted the apoptosis of JEG-3 cells. Notably, lncRNAs act as sponges of miRNAs and compete for complementary binding with miRNAs, thereby inhibiting their function (30). Previous studies have reported that NEAT1 regulated cell motility by targeting miR-361-5p (31), miR-101-3p (32) and miR-37 (33). In this research, miR-125b was identified as a direct target of NEAT1 and was also negatively regulated by NEAT1. Therefore, it was hypothesized that miR-125b may interact with NEAT1 to modulate RSA progression. Rescue experiments confirmed that upregulation of miR-125b reversed the enhancing effect of NEAT1 overexpression on cell viability, and the inhibitory effect on apoptosis further validated this assumption.

During early pregnancy, both fetal and maternal tissues experience cell death caused by apoptosis and susceptivity to RSA is associated with the balance of cell death and proliferation (34). BCL-2, an anti-apoptotic protein, plays a key role in regulating endometrial cell turnover (35). A recent study reported that BCL-2 expression was reduced in trophoblastic tissues of patients with RSAs (36). Similarly, a reduction in BCL-2 expression was discovered in the villi of patients with RSAs, suggesting that low BCL-2 expression may be associated with RSA. Previous studies have demonstrated that overexpression of BCL-2 can enhance cell viability and suppress apoptosis and miRNAs such as miR-34a (37) and miR-195-5p (38) regulated this effect by targeting BCL-2. In the current study, BCL-2 was identified as a downstream target gene of miR-125b. Based on previous data showing that NEAT1 overexpression attenuated the malignant behavior of RSA by regulating miR-125b, it was hypothesized that BCL-2 may be involved in RSA pathogenesis by modulating the NEAT1/miR-125b axis. Transfection of sh-BCL-2 significantly reversed the enhancing effect of pcDNA-NEAT1 on cell viability and the suppressive effect on apoptosis in JEG-3 cells. In conclusion, NEAT1 overexpression enhanced the viability and inhibited the apoptosis of JEG-3 cells by interacting with the miR-125b/BCL-2 axis.

There are several limitations in the present study. First, the effects of the NEAT1/miR-125b/BCL-2 axis on biological processes such as cell migration and invasion, in addition to cell viability and apoptosis, need to be investigated in RSA.

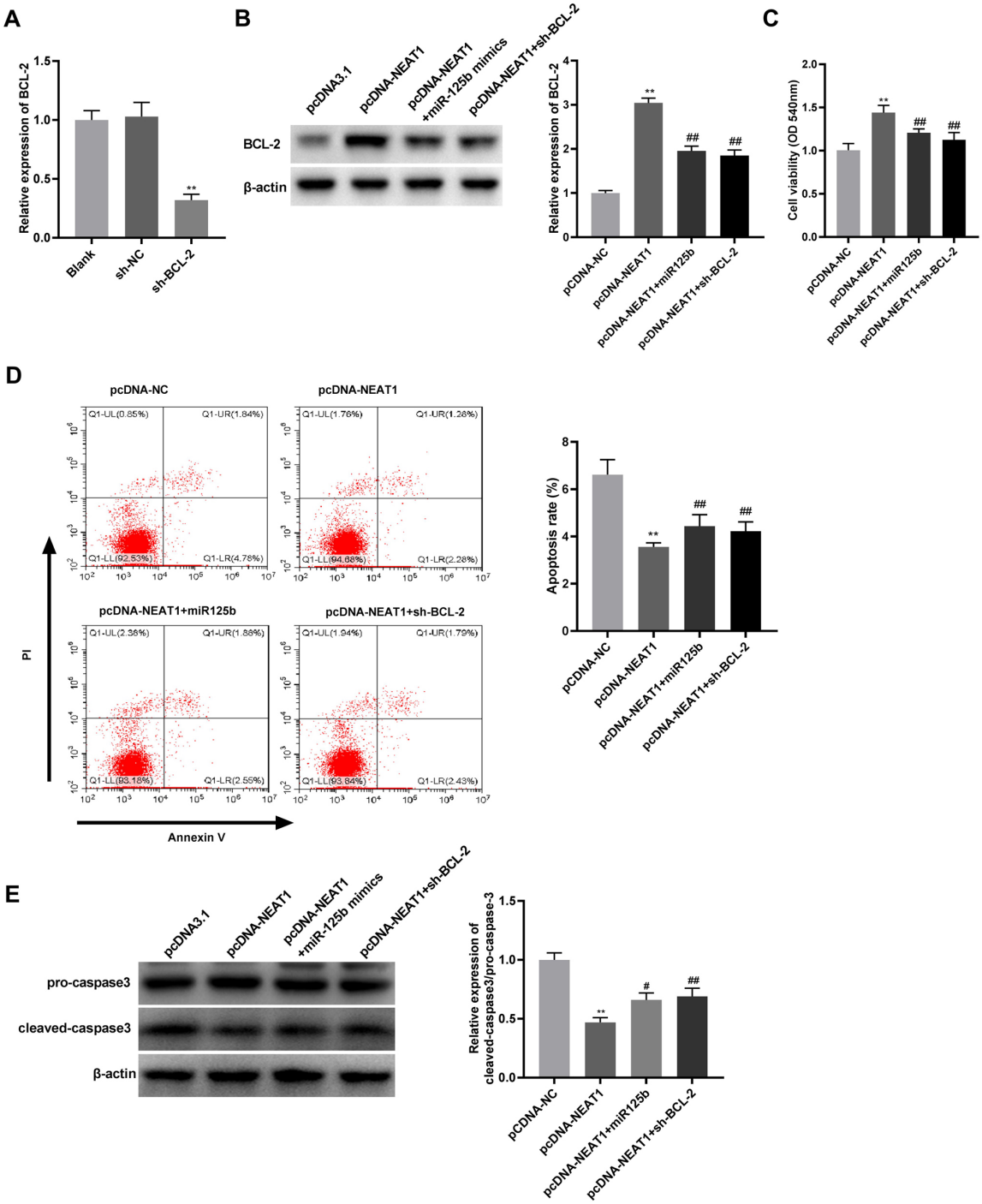

Figure 5. NEAT1 overexpression promotes cell viability and inhibits apoptosis by regulating miR-125b/BCL-2 axis. (A) The expression of BCL-2 was measured by RT-qPCR in JEG-3 cells transfected with sh-BCL-2 or sh-NC. **P<0.01 vs. sh-NC. (B) The protein expression of BCL-2 was detected by western blot analysis in JEG-3 cells transfected with pcDNA-NC, pcDNA-NEAT1, pcDNA-NEAT1 + miR-125b mimics or pcDNA-NEAT1 + sh-BCL-2. **P<0.01 vs. pcDNA-NC; $^{\#\#}$P<0.01 vs. pcDNA-NEAT1. (C) Cell viability was detected by MTT assay in JEG-3 cells transfected with pcDNA-NC, pcDNA-NEAT1, pcDNA-NEAT1 + miR-125b mimics or pcDNA-NEAT1 + sh-BCL-2. **P<0.01 vs. pcDNA-NC; $^{\#\#}$P<0.01 vs. pcDNA-NEAT1. (D) Flow cytometry was used to detect the apoptotic rates of JEG-3 cells transfected with pcDNA-NC, pcDNA-NEAT1, pcDNA-NEAT1 + miR-125b mimics, or pcDNA-NEAT1 + sh-BCL-2. **P<0.01 vs. pcDNA-NC; $^{\#\#}$P<0.01 vs. pcDNA-NEAT1. (E) Ratio of protein expression of cleaved caspase3/pro-caspase 3 was detected by western blot analysis in JEG-3 cells transfected with pcDNA-NC, pcDNA-NEAT1, pcDNA-NEAT1 + miR-125b mimics or pcDNA-NEAT1 + sh-BCL-2. **P<0.01 vs. pcDNA-NC; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. pcDNA-NEAT1. NEAT1, nuclear paraspeckle assembly transcript 1; miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR; sh, short hairpin; NC, negative control.

Second, the interactions between this regulatory axis and relevant downstream signaling pathways remain unclear. Third, the present study only focused on elucidating the mechanism behind the NEAT1/miR-125b/BCL-2 axis at the cellular level and *in vivo* experiments will be required to supplement the present results. Further studies are required in order to address these issues.

In summary, the present study revealed that NEAT1 expression was reduced in the villi of patients with RSAs and that NEAT1 overexpression regulated the viability and apoptosis

of JEG-3 cells by targeting the miR-125b/BCL-2 axis. These findings offer new insights into the etiology of RSA and may aid in identifying potential targets for RSA treatment.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XL and HZ are mainly responsible for the design of articles, data analysis, methodology, project management and modification of important contents and drafting of manuscripts. LS, BX and JL are responsible for resource integration, experimental data analysis, software, visualization, investigation and literature query, manuscript modification and editing. All authors have been involved in writing, editing, reading and approving the current version. All authors confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Liaocheng Dongchangfu Maternal and Child Health Hospital (Liaocheng, China; approval ID: 2020-02) and all participants undersigned the informed consents.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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