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Original Article

LncRNA CRNDE regulates trophoblast cell proliferation, invasion, and migration via modulating miR-1277

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Abstract: Preeclampsia (PE) is a pregnancy-specific syndrome and contributes to maternal and perinatal morbidity and mortality, but the underlying mechanisms of PE remain indistinct. This study aims to investigate the functional role of LncRNA CRNDE in PE pathogenesis and its effects on trophoblasts. mRNA levels of CRNDE and miR-1277 in placenta tissues and HTR-8/SVneo cells were analyzed by qRT-PCR. Western blot was carried out to evaluate protein level of E-cadherin, β-Catenin, Vimentin, MMP2 and MMP9. Cell proliferation was assessed by using CCK-8 assay and colony formation assay, whereas cell migration and invasion were evaluated by wound healing assay and transwell assay, respectively. Moreover, the interrelation between CRNDE and miR-1277 was verified by dual luciferase reporter assay. The results showed that CRNDE expression was significantly downregulated, whereas miR-1277 expression was significantly upregulated in PE placental tissues. Overexpression of CRNDE facilitated HTR-8/SVneo cell proliferation, migration and invasion, promoted the EMT formation, and increased the protein expression of MMP2 and MMP9, while knockdown of CRNDE had the opposite results. In addition, we also found that miR-1277 was negatively regulated by CRNDE and was a direct target of CRNDE. Furthermore, knockdown of miR-217 promoted HTR-8/SVneo cell proliferation, migration, and invasion, while knockdown of CRNDE reversed the accelerating effects of miR-1277 on HTR-8/SVneo cells. LncRNA CRNDE may suppress trophoblast cell proliferation, migration and invasion at least partly through regulating miR-1277 in trophoblast cells. CRNDE is promising to function as a new target for intervention of PE.

Keywords: Preeclampsia (PE), CRNDE, miR-1277, trophoblast, proliferation, metastasis

Introduction

Preeclampsia (PE), characterized by new-onset hypertension and proteinuria, is one of the main causes of maternal and neonatal high morbidity and mortality [1-3]. Previous studies have reported that many factors are involved in the pathogenesis of PE, such as impaired spiral artery remodeling, oxygen dysregulation, systemic inflammation and inappropriate maternal vascular destruction [4, 5]. Among these factors, pool spiral artery remodeling is considered to be a critical inducement causing PE and is associated with dysfunctions of extravillous trophoblasts (EVTs) [1, 6]. Under normal condition, trophoblasts give nutrition supply to the fetus by the invasion of the maternal spiral arteries, as well as the migration through the decidua and myometrium. In PE, the abnormal EVTs cannot invade the myometrium, leading to placental hypoperfusion and impairment of spiral artery remodeling [7].

Long noncoding RNAs (lncRNAs) are nonprotein-coding transcripts that are more than 200 nucleotides [8]. lncRNAs take part in diverse biological process such as cell proliferation, metastasis and oxidative stress [9]. Growing evidence has shown that lncRNAs serve as regulatory factors in the occurrence and progress of PE. Previous studies revealed that lncRNA TUG1 inhibited the proliferation of trophoblast cells through binding to EZH2 and the level of TUG1 was significantly decreased in PE compared to the normal [10]. In addition, lncRNA ATB level was shown to be declined in the early-onset preeclampsia and the downregulation of ATB inhibited the proliferation, invasion and tube formation abilities in HTR-8/SVneo [11]. Furthermore, the expression of lncRNA SPRY4-

IT1 was proved to increase in PE placenta, and SPRY4-IT1 suppressed trophoblast cell migration and invasion by mediating the Wnt/β-catenin pathway associated with EMT [12]. LncRNA colorectal neoplasia differentially expressed (CRNDE) was found to play a critical role in the progression of various cancers, such as hepatic carcinoma, ovarian cancer and gallbladder carcinoma [13-15]. However, the functional role of LncRNA CRNDE in trophoblast is still indistinct.

In the study, we investigated the regulatory function of CRNDE on trophoblast cell proliferation, invasion and EMT. Furthermore, we elucidated the potential mechanisms by which CRNDE regulates the pathogenesis of PE. Our results provided a new understanding of biological role of CRNDE in the progression of PE.

Materials and methods

Clinical samples and patients

We collected 30 paired placenta tissues from PE women and normal pregnancies that were primipara and underwent cesarean deliveries at Shandong Provincial Hospital Affiliated to Shandong University in 2017-2018. After washing with PBS, all the placental tissues were stored at -80°C. Severe PE was made a definite diagnosis according to the definition in Williams Obstetrics (23rd edition) [16]. All the subjects had no chronic nephritis, diabetes, smoking, vaginal delivery, heart diseases, autoimmune diseases, cancer and hemostatic abnormalities. Experiments were approved by the Ethics Committee of Maternal and Child Health Care Hospital of Shandong Province, and the informed consents were obtained from each patient.

Cell culture

The human trophoblast cell line HTR-8/SVneo was obtained from ATCC compony (Manassas, VA). Cells were cultured in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, CA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific), 100 μg/mL streptomycin, and 100 U/mL penicillin in a humidified atmosphere at 37°C with 5% CO_2.

Cell transfection

pcDNA-CRNDE, shRNA-CRNDE, WT-CRNDE, Mut-CRNDE, miR-1277 mimic, miR-1277 inhibitor and negative controls were provided by GenePharma (Shanghai, China). The recombinants and miRNAs mimics or inhibitors were transfected into HTR-8/SVneo cells by Lipofectamine 2000 reagent (Invitrogen, CA). After 48 hours, cells were collected for the following experiments.

Quantitative real time-PCR (qRT-PCR)

Total RNA was isolated from placental tissues and HTR-8/SVneo cells using Trizol Reagent (Invitrogen). miR-1277 and CRNDE expression were measured by using the SYBR Premix Ex Taq II Kit (Takara, Japan). qRT-PCR was performed with an ABI7900 system (Applied Biosystems, MA). The PCR amplification program was at an initial denaturation step of 1 min at 94°C, followed by 40 cycles of 95°C for 30 sec and 60°C for 1 min. GAPDH and U6 were chosen as the internal controls for CRNDE and miR-1277, respectively.

Cell Counting Kit-8 assay

Proliferation viability of HTR-8/SVneo cells was assessed by Cell Counting Kit-8 (CCK-8) assay according to the manufacturer's instructions. Briefly, after transfection for 24, 48 and 72 hours, 10 μL of CCK-8 reagent (Beyotime) was added into each well for 2.5 hours. Then the absorbance at a wavelength of 450 nm was measured using a microplate reader (Bio-Tek Instruments, VT). Each group was replicated for five times and the assay was conducted independently thrice.

Colony formation assay

Cells were seeded in a six-well plate at a density of 2×10^4 cells/well and transfected with pcDNA-CRNDE or shRNA-CRNDE for 48 hours. Two weeks later, the cells were washed with PBS, fixed with ethanol for 30 min and stained with 1% crystal violet for counting colony number. Three independent assays were performed.

Wound healing assay

The cell migration ability was investigated by wound healing assay. Cells were seeded in a six-well plate at 2×10^4 cells per well, transfected, and cultured until 80-90% confluence. A scratch through the cell monolayers was created by a pipette tip and the debris was removed with phosphate-buffered saline. After incuba-

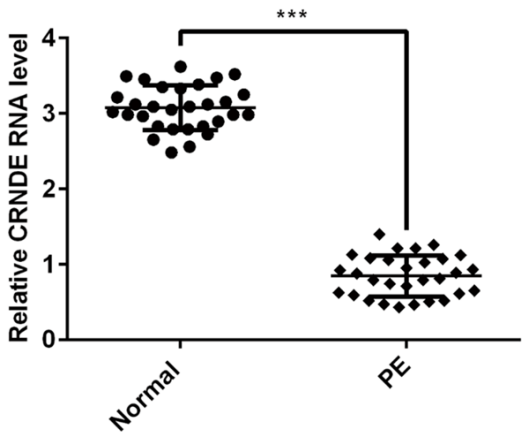


Figure 1. CRNDE is significantly decreased in placenta tissues with PE. The relative expression of lncRNA CRNDE was measured by qRT-PCR. GAPDH was used as an internal reference. Data are expressed as mean ± SD. ***P<0.001 versus the control.

tion for 24 hours, images were obtained and the numbers of migrated cells were counted with an inverted microscope (Nikon, Tokyo, Japan). Each experiment was carried out in triplicate.

Cell invasion assays

The invasion capacity of the trophoblast cells was assessed by transwell assay. The transwell chambers (6.5 mm in diameter, 8 μm pore-size, Corning Costar, Cambridge, MA) were coated with 0.1 mL of matrigel (BD Bioscience, Franklin Lakes, NJ, and USA) at 37°C overnight for gelling. Then, the transfected cells were re-suspended and seeded into the upper chamber at a density of 5×10^4 cells/well. Meanwhile, RMPI 1640 medium with 10% FBS was added into the lower chamber. After 24 h incubation at 37°C in 5% CO_2, the noninvasive cells on upper side were wipe off with cotton swabs. The invasive cells were fixed with 4% paraformaldehyde for 10 minutes, and stained with 0.5% crystal violet at room temperature for 10 minutes. The number of invasive cells was observed with a light microscope. Five randomly chosen fields were counted for each group and all assays were independently repeated three times.

Western blot assay

Proteins from HTR-8/SVneo cells were extracted using mammalian reagent RIPA (Beyotime, China). The concentration of the proteins in each sample was examined by using the BCA Assay Kit (Beyotime, China). Proteins from each sample were separated by SDS-PAGE and electrophoretically transferred onto a PVDF membrane (Invitrogen, USA). The membrane was then blocked with 5% skimmed milk at room temperature for 1 hour and incubated at 4°C overnight with primary antibodies (Anti-E-Cadherin, 1:50, Abcam; Anti-β-Catenin, 1:4000, Abcam; Anti-Vimentin, 1:100, Abcam; Anti-MMP2, 1 μg/ml, Abcam; Anti-MMP9, 1:1000, Abcam; Anti-GAPDH, 1 μg/ml, Abcam). Afterwards, the membrane was incubated with goat anti-rabbit or mouse IgG H&L secondary antibodies (Abcam) for 2 hours. The target protein expressions were analyzed by using the ECL Detection System (Life technologies, Gaithersburg). Each experiment was carried out three times.

Dual luciferase reporter assay

The sequence from CRNDE containing the predicted miR-1277 binding site was amplified, and then cloned into a pMIR-REPORT Dual-luciferase miRNA Target Expression Vector (Ambion) to generate the CRNDE reporter (WT-CRNDE). Mutations in CRNDE mRNA were created using the QuickChange Site-Directed Mutagenesis kit (Stratagene). Thereafter, the reporter vectors and miR-1277 mimic or negative control (miR-NC) were cotransfected into cells by the Lipofectamine 2000 transfection reagent (Invitrogen). Finally, the Luciferase reporter activity was detected by a dual luciferase reporter assay (Promega, Madison, WI, USA).

Statistical analysis

SPSS 20.0 soft was used to perform statistical analyses. Differences between two groups were assessed using the Student's t-test (two-tailed). Multiple comparisons were analyzed using one-way analysis of variance (ANOVA). Data were presented as the mean ± SD. P<0.05 was considered to indicate statistical significance.

Results

Expressions of CRNDE and miR-1277 in human placenta tissues

Firstly, we examined the expression level of CRNDE in 30 pairs of placental tissues with severe PE by qRT-PCR. As presented in **Figure 1**, we found that CRNDE expression was significantly downregulated in placenta tissues with severe PE than the normal tissues (**Figure 1**).

Upregulation of CRNDE promotes trophoblast proliferation, metastasis and the EMT formation

To further explore the biological function of CRNDE in trophoblasts, we transfected HTR-8/SVneo cells with CRNDE-overexpressing vector (pcDNA-CRNDE) or empty vector (pcDNA-NC) and the CRNDE mRNA expression was measured by qRT-PCR. The results revealed that the expression of CRNDE was remarkably elevated in cells transfected with CRNDE-overexpressing vector compared with the control (**Figure 2A**). The CCK-8 assay and colony formation assay showed that CRNDE overexpression promoted the cell growth and increased the colony number in the pcDNA-CRNDE group when compared with the control (**Figure 2B** and **2C**). In addition, transwell assay and wound healing assay were employed to detect the invasion and migration capacity, respectively. The data showed that CRNDE overexpression obviously enhanced the capacity of migratory and invasive cells (**Figure 2D-G**). At the same time, we also evaluated the protein levels of MMP2 and MMP9. Consistently, increased expressions of MMP2 and MMP9 were observed in pcDNA3.1-CRNDE transfected cells (**Figure 2H**). Finally, western blot analysis was used to determine EMT of HTR-8/SVneo cells. As shown in **Figure 2I**, CRNDE overexpression reduced the expressions of E-cadherin and β-catenin whereas increased expression of Vimentin in HTR-8/SVneo cells.

Knockdown of CRNDE suppresses trophoblast proliferation, invasion, migration and affected the EMT formation

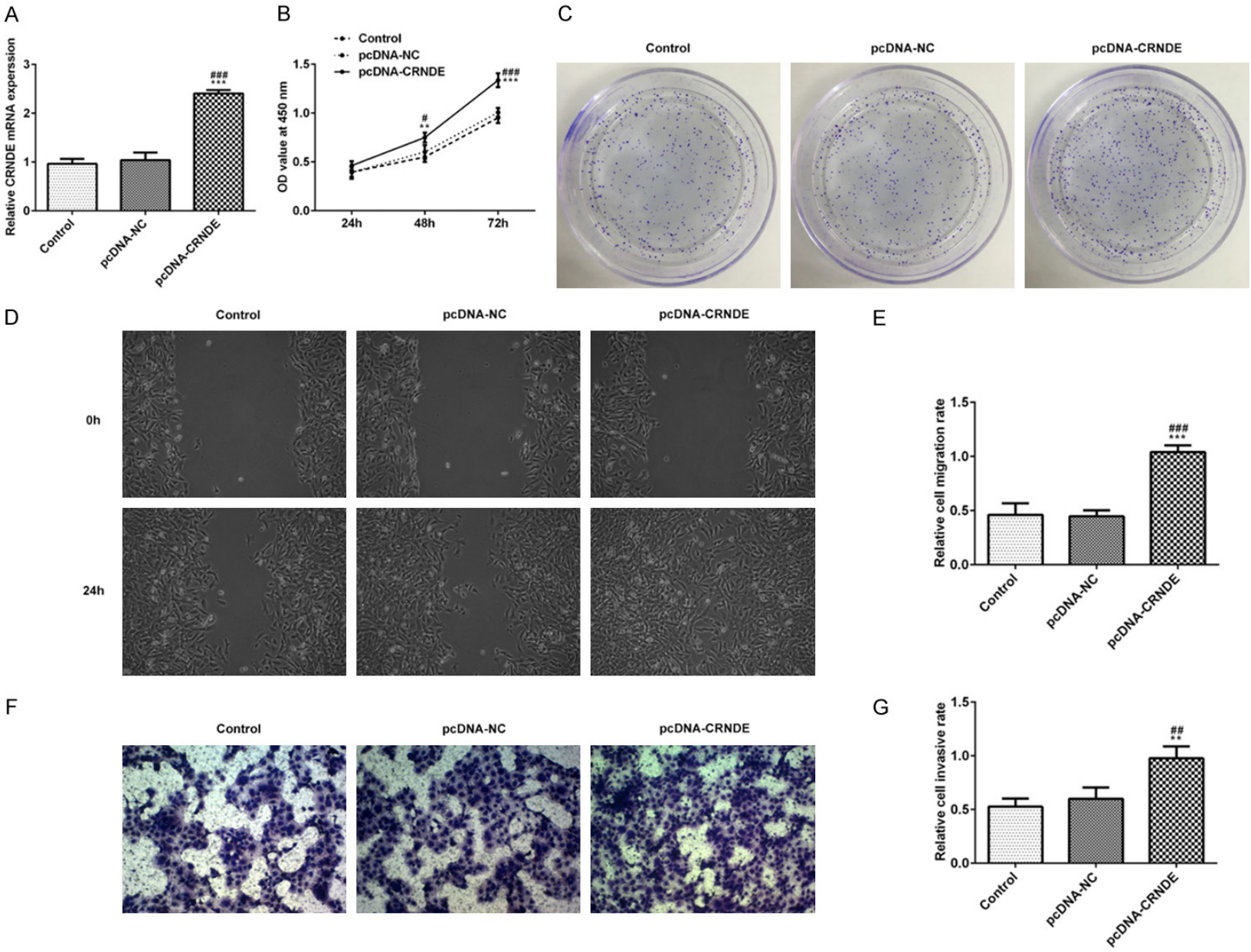
Then, we transfected the interfering CRNDE (shRNA-CRNDE) into HTR-8/SVneo cells to obtain the downregulation of CRNDE. The qRT-PCR result showed that shRNA-CRNDE significantly repressed the CRNDE expression compared with the control (**Figure 3A**). As presented in **Figure 3B**, knockdown of CRNDE extremely suppressed the cell proliferative capacity of HTR-8/SVneo cells. The colony number in HTR-8/SVneo cells transfected with shRNA-CRNDE was significantly decreased, which is consistent with the result above (**Figure 3C**). The results from transwell assay and wound healing assay showed that CRNDE knockdown evidently attenuated the cell invasive and migratory ability compared with the control (**Figure 3D-G**). Moreover, the protein expressions of MMP2 and MMP9 were found to be reduced after transfection with shRNA-CRNDE (**Figure 3H**). According to western blotting assay, CRNDE silence markedly increased protein levels of E-cadherin and β-catenin, and decreased Vimentin level in contrast to the control group (**Figure 3I**).

CRNDE directly targets miR-1277

To predict the miRNAs potentially binding to the 3'UTR of CRNDE, we used the bioinformatics analysis and found that miR-1277 contained the CRNDE binding sequence. We then preformed dual luciferase reporter assay to confirm whether miR-1277 harbored the complementary sequences with CRNDE. The luciferase reporter vectors were constructed, which contain the wild-type and mutated sequences of CRNDE (**Figure 4A**). Upregulation of miR-1277 dramatically weakened the luciferase activity of cells co-transfected with a luciferase vector containing wild-type CRNDE segment and miR-1277 mimic. However, the luciferase activity of HTR-8/SVneo cells with the mutant reporter vector was unchanged (**Figure 4B**). The expression level of miR-1277 in HTR-8/SVneo cells was markedly decreased when CRNDE was upregulated compared with the control (**Figure 4C**), while CRNDE knockdown displayed the opposite results (**Figure 4D**). Additionally, we discovered a significant increase of miR-1277 in the PE tissues than the control group according to the analysis of the tissue samples (**Figure 4E**). Additionally, CRNDE in the PE tissues was negatively correlated with the expression of miR-1277 ($r=-0.4476$, $P=0.0131$; **Figure 4F**).

Knockdown of miR-1277 modulates proliferation, invasion, migration and EMT of HTR-8/SVneo cell

To investigate the functional role of miR-1277 in PE, we achieved the downregulation of miR-1277 by transfecting with miR-1277 inhibitors. The result from qRT-PCR showed that mRNA level of miR-1277 in HTR-8/SVneo cells was significantly lower than the control group (**Figure 5A**). CCK-8 assay showed that the HTR-8/SVneo cell proliferation was promoted in the miR-1277 inhibitor group and the effect was offset with the addition of shRNA-CRNDE (**Figure 5B**). In addition, the colony number in miR-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Relative CRNDE mRNA expression
Control
pcDNA-NC
pcDNA-CRNDE
B
OD value at 450 nm
24h
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Relative cell migration rate
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Relative cell invasive rate

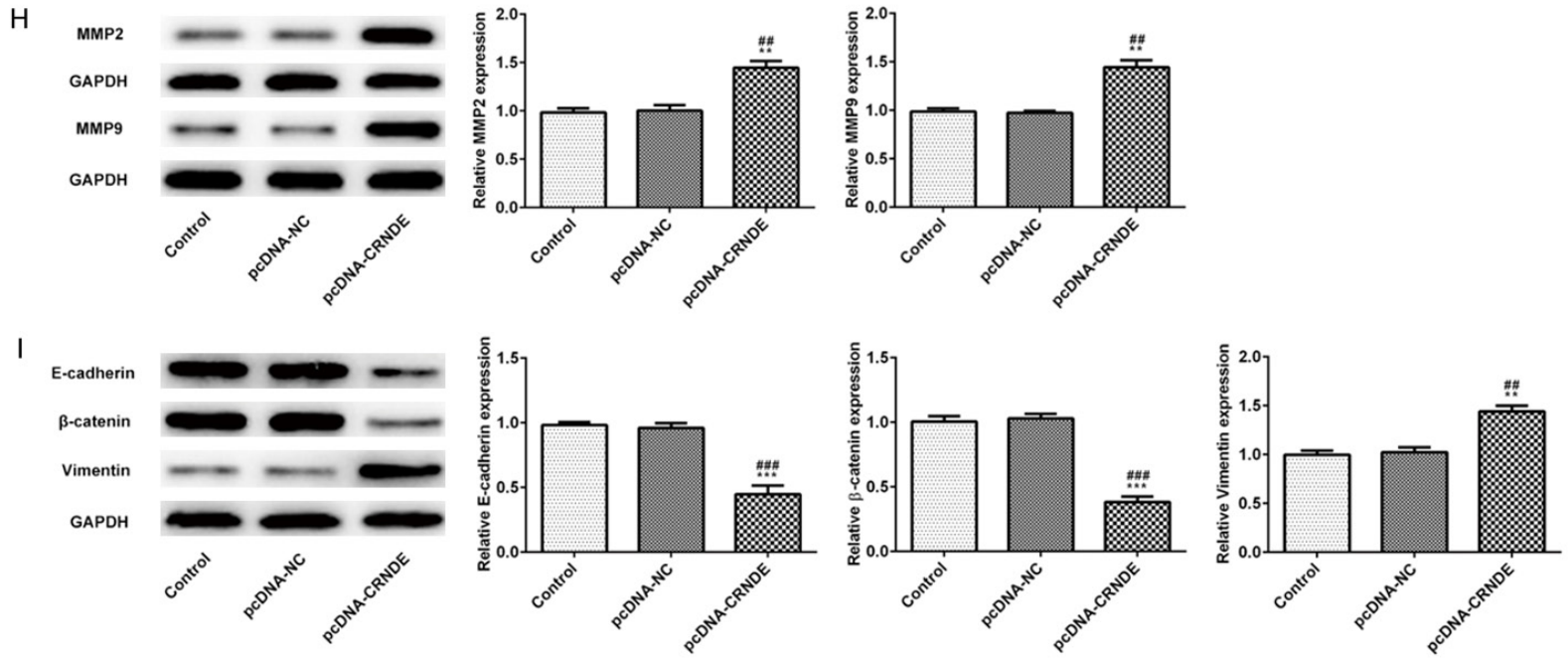
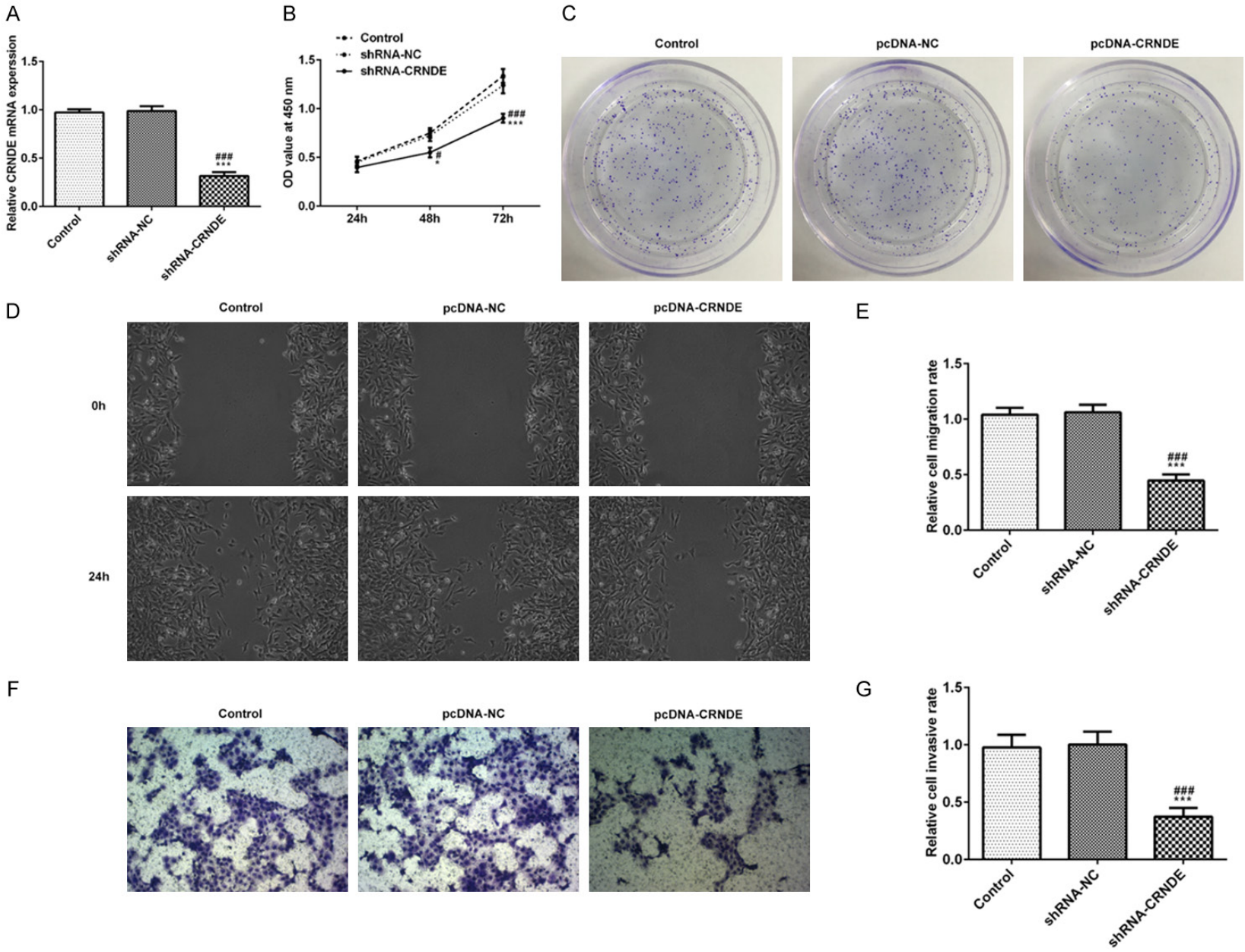


Figure 2. CRNDE overexpression promotes trophoblast proliferation, invasion, migration and EMT formation. A. The expression level of CRNDE in HTR-8/SVneo cells transfected with pcDNA-CRNDE or pcDNA-NC was evaluated by qRT-PCR. B and C. The proliferation ability was determined with CCK-8 assay and colony formation assay in HTR-8/SVneo cells after being transfected with pcDNA-CRNDE or pcDNA-NC. D-G. Wound healing assay and invasion assay were employed to detect cell migration and invasion in HTR-8/SVneo cells after transfection with pcDNA- CRNDE or pcDNA-NC. Original magnification, 100×. H and I. Western blot assay was performed in HTR-8/SVneo cells transfected with pcDNA-CRNDE or pcDNA-NC to evaluate the levels of MMP2, MMP9 and proteins involved in EMT. GAPDH was used as an internal reference. Data are expressed as mean ± SD. **P<0.01, ***P<0.001 versus the control; $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 versus pcDNA-NC.


A
Relative CRNDE mRNA expersion
1.5
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Control
shRNA-NC
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B
Control
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shRNA-CRNDE
OD value at 450 nm
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1.0
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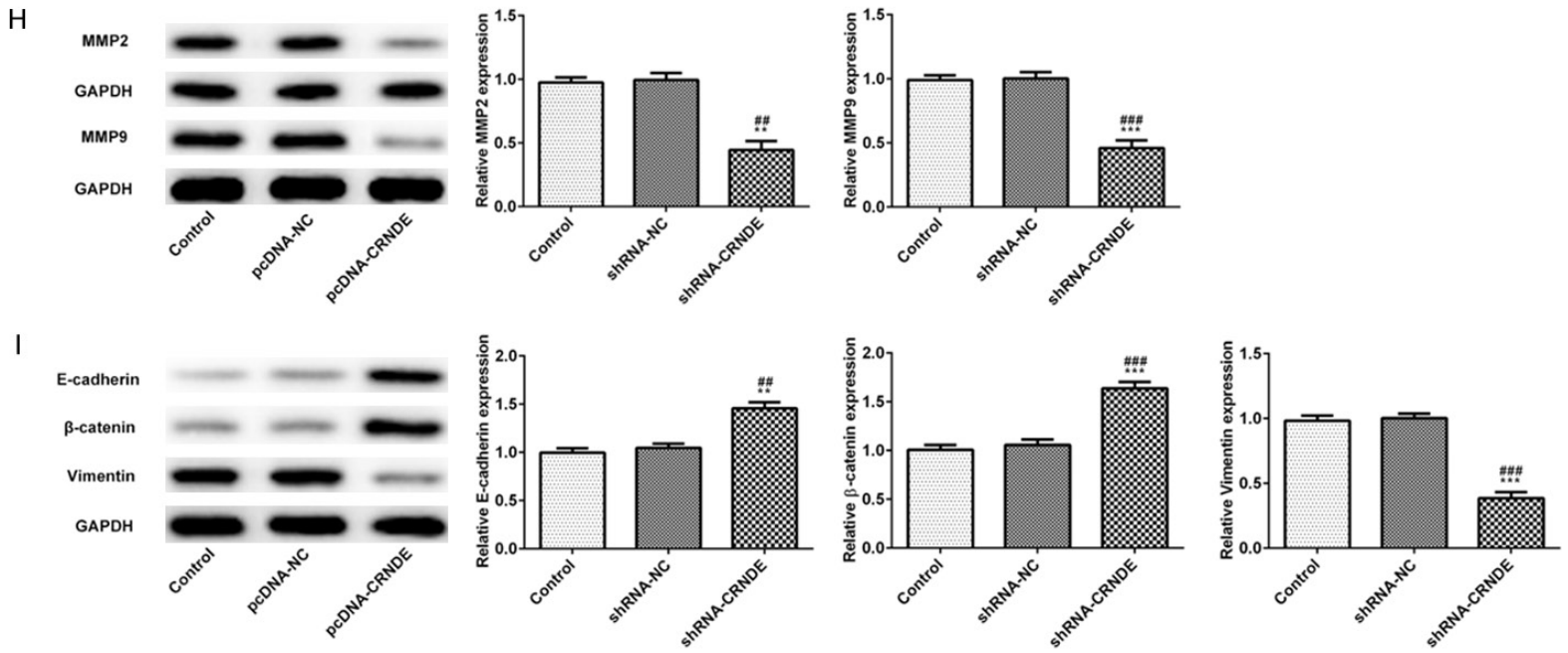


Figure 3. CRNDE knockdown suppresses trophoblast proliferation, invasion, migration and EMT formation. A. The expression level of CRNDE in HTR-8/SVneo cells transfected with shRNA-CRNDE or shRNA-NC was evaluated by qRT-PCR. B and C. The proliferation ability was determined with CCK-8 assay and colony formation assay in HTR-8/SVneo cells after being transfected with shRNA-CRNDE or shRNA-NC. D-G. Wound healing assay and invasion assay were carried out in trophoblast cells transfected with shRNA-CRNDE or shRNA-NC to detect cell migration and invasion. Original magnification, 100×. H and I. Western blot assay was performed in HTR-8/SVneo cells transfected with shRNA-CRNDE or shRNA-NC to analyze the levels of MMP2, MMP9 and proteins involved in EMT. GAPDH was used as an internal reference. Data are expressed as mean ± SD. **P<0.01, ***P<0.001 versus the control; $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 versus shRNA-NC.

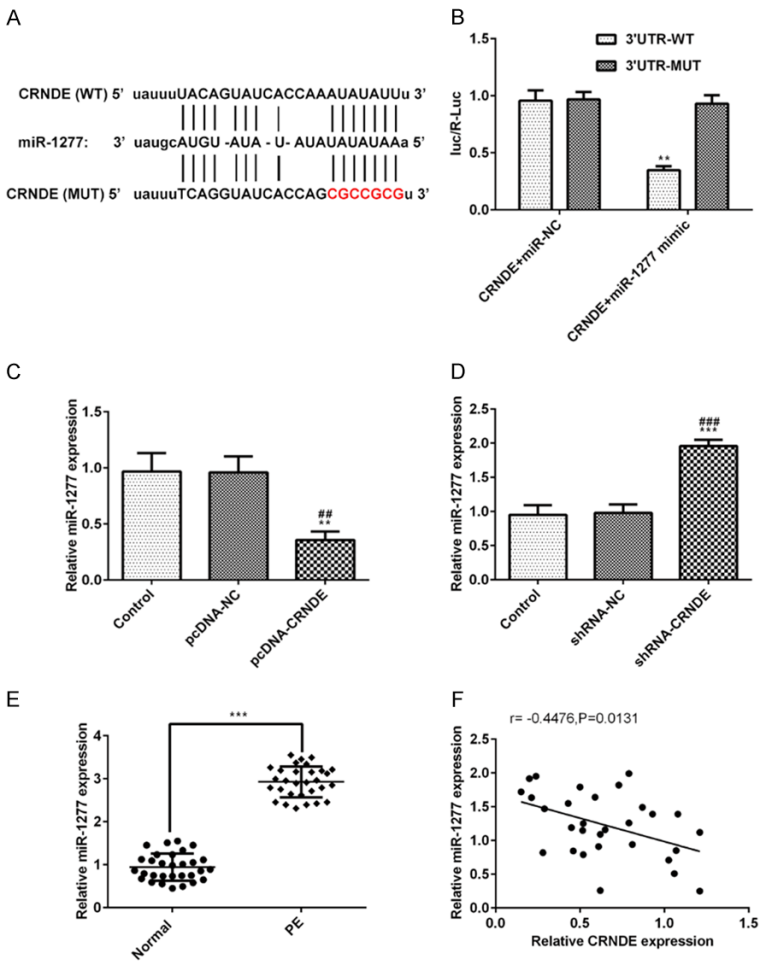


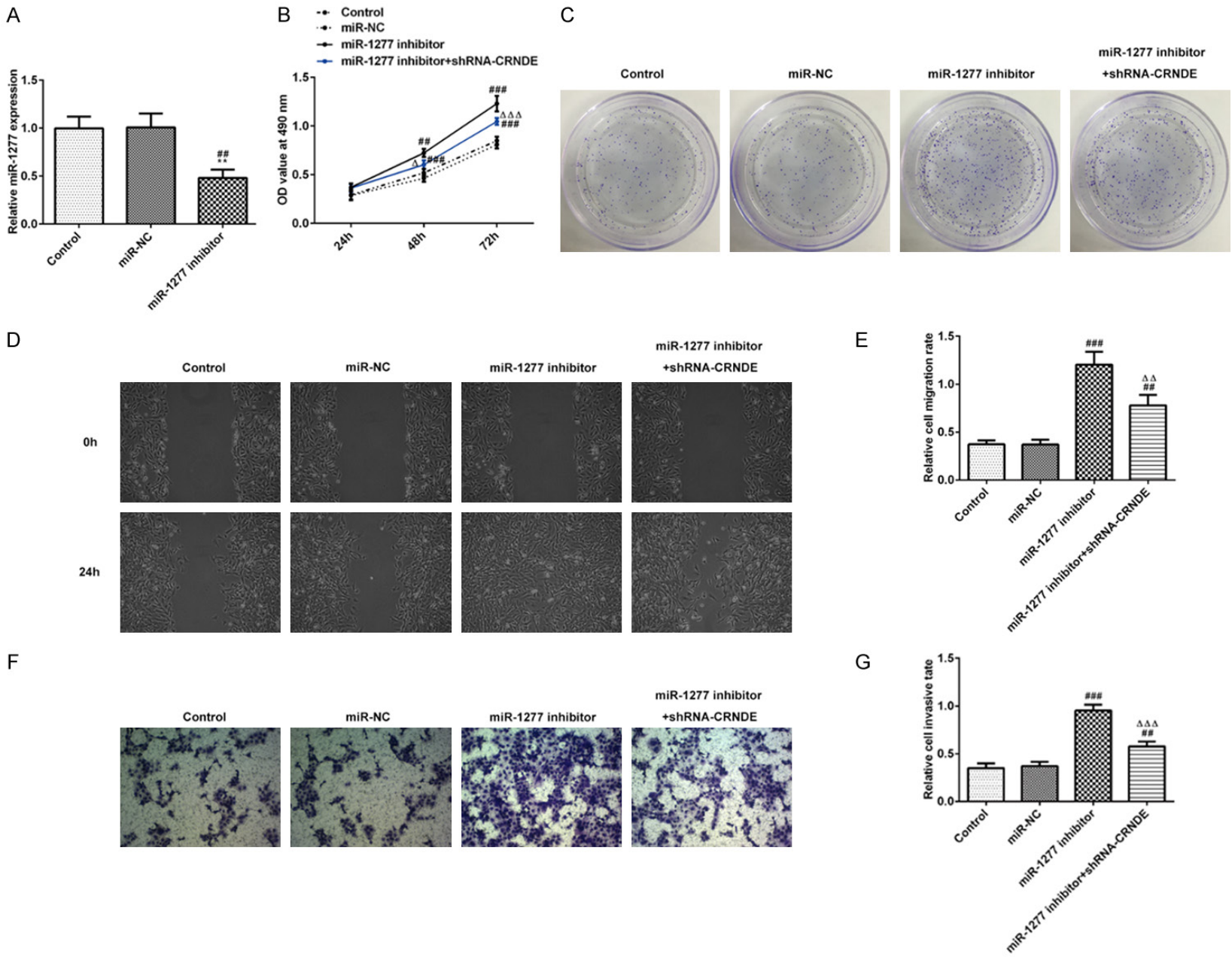
Figure 4. CRNDE interacts with miR-1277 in trophoblast. A. The predictive binding site between CRNDE and miR-1277. B. Luciferase reporter assay in HTR-8/SVneo cells cotransfected with miR-1277 mimic and wild-type/mutant CRNDE was used to confirm the luciferase activity. C. qRT-PCR was implemented to measure the relative expression level of miR-1277 in pcDNA-CRNDE or pcDNA-NC transfected HTR-8/SVneo cells. D. The relative expression level of miR-1277 in HTR-8/SVneo cells transfected with shRNA-CRNDE or shRNA-NC was measured by using qRT-PCR. E. qRT-PCR was performed to determine the relative expression of miR-1277 in 30 pairs of PE placenta tissues and normal placenta tissues. F. The correlation between CRNDE and miR-1277 expression in PE and normal placental tissues was estimated by Spearman's correlation analysis. Data are expressed as mean ± SD. **P<0.01, ***P<0.001 versus the control; $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 versus respective NC groups.

1277 inhibitor transfected cells was extremely increased compared with the control and shRNA-CRNDE reversed the quantity (**Figure 5C**). The transwell assay and wound healing assays showed that the capabilities of the invasion and migration were significantly elevated after transfected miR-1277 inhibitor and the elevated capability was weakened by shRNA-CRNDE (**Figure 5D-G**). Furthermore, we observed increased expressions of MMP2 and MMP9 in miR-1277 inhibitor group when compared with the miR-NC group whereas shRNA-CRNDE attenuated the accelerated effect of miR-1277 inhibitor (**Figure 5H**). Besides, downregulated miR-1277 observably reduced the protein levels of E-cadherin and β-catenin, and increased Vimentin level, while shRNA-CRNDE reversed the tendency (**Figure 5I**).

Discussion

Preeclampsia contributes to high fetal morbidity and pregnancy-induced mortality [17]. Its complex pathogeny and risky syndromes in clinic increase the difficulty of treatment and nursing care for PE patients. Growing evidence has shown that lncRNAs play important roles in the pathogenesis and development of PE [18-20]. In the current study, we identified that CRNDE expression was notably downregulated, whereas miR-1277 expression was upregulated in placenta tissues. CRNDE overexpression promoted trophoblast proliferation, metastasis and EMT formation, while CRNDE silencing exhibited the opposite effects. The bioinformatics analysis showed that miR-1277 was the direct target of CRNDE and was regulated negatively by CRNDE in the trophoblasts. Furthermore, we demonstrated that miR-1277 knockdown promoted trophoblast proliferation, metastasis and EMT formation, while CRNDE knockdown attenuated the stimulative effects of miR-1277 knockdown. Thus, CRNDE may exert its biological functions via regulating miR-1277 expression.

Increasing evidence has demonstrated that CRNDE plays an important role in different biological processes, especially in various types of cancers [21, 22]. Han et al showed that CRNDE


A
Relative miR-1277 expression
1.5
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##
Control
miR-NC
miR-1277 inhibitor
B
Control
miR-NC
miR-1277 inhibitor
miR-1277 inhibitor+shRNA-CRNDE
OD value at 490 nm
1.5
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miR-1277 inhibitor
miR-1277 inhibitor
+shRNA-CRNDE
D
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miR-1277 inhibitor
miR-1277 inhibitor
+shRNA-CRNDE
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Relative cell migration rate
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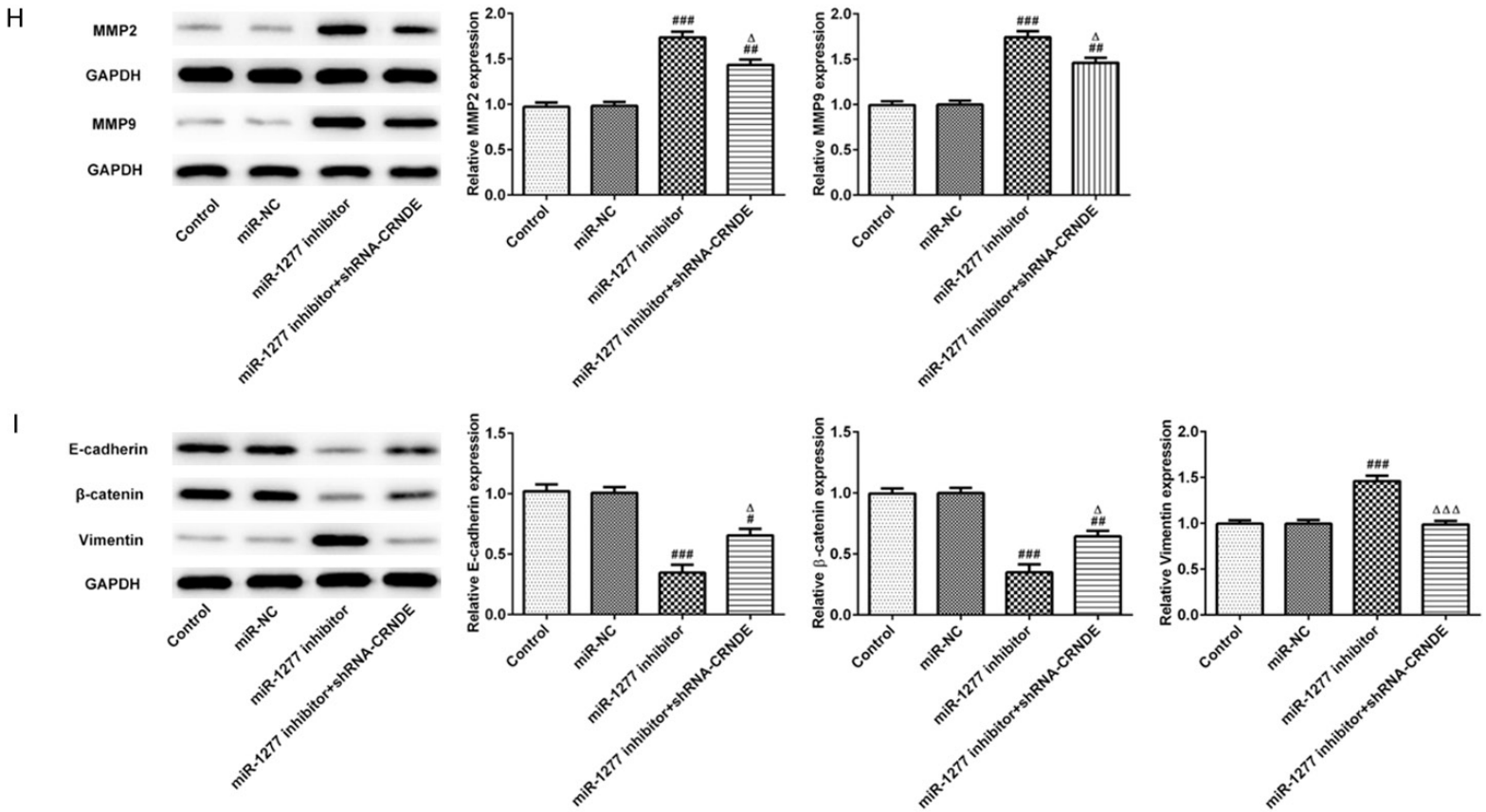

Figure 5. MiR-1277 silence promotes trophoblast proliferation, invasion, migration and EMT formation. A. The expression level of miR-1277 in miR-1277 inhibitor or miR-NC transfected HTR-8/SVneo cells was evaluated by qRT-PCR. B and C. The proliferation ability in HTR-8/SVneo cells transfected with miR-1277 inhibitor and shRNA-CRNDE was determined by CCK-8 assay and colony formation assay. D-G. Wound healing assay and invasion assay were employed to detect cell migration and invasion in HTR-8/SVneo cells transfected with miR-1277 inhibitor and shRNA-CRNDE. Original magnification, 100×. H and I. Western blot assay was applied for determining the levels of MMP2, MMP9 and proteins involved in EMT in HTR-8/SVneo cells transfected with miR-1277 inhibitor and shRNA-CRNDE. GAPDH was used as an internal reference. Data are expressed as mean ± SD. **P<0.01 versus the control; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 versus miR-NC; $^{\Delta}$P<0.05, $^{\Delta\Delta}$P<0.01, $^{\Delta\Delta\Delta}$P<0.001 versus miR-1277 inhibitor.

regulated the proliferation and chemoresistance of colorectal cancer cell via modulating miR-181a-5p expression [23]. Zheng et al reported that overexpression of CRNDE promoted proliferation, migration, and inhibited the apoptosis in glioma stem cells, while knockdown of CRNDE reduced tumor formation rate in tumor-bearing nude mice [24]. Additionally, Wang et al revealed that CRNDE expression was increased in glioma stem cells and CRNDE overexpression promoted glioma cell growth and invasion in vitro and in vivo [25]. Shao et al found that CRNDE was highly expressed in renal cell carcinoma malignant tissues and upregulation of CRNDE accelerated RCC cell proliferation through activation of Wnt/β-catenin signaling [26]. However, the effect of CRNDE in PE hasn't been investigated. In the present study, we confirmed a significant reduction of CRNDE in placental tissues compared with the normal tissues. CRNDE overexpression promoted trophoblast cell proliferation, invasion, migration, and even facilitated the EMT formation, which is consistent with previous studies. These results suggested that CRNDE may be a vital mediator in the process of PE.

lncRNAs have shown function roles in human microenvironment through interaction with microRNA [27-29]. In this study, we found that miR-1277 expression was significantly downregulated in placenta tissues and its expression was suppressed by CRNDE in HTR-8/SVneo cells. In addition, CRNDE negatively regulated the expression of miR-1277 in the placenta tissues with PE. Previous study showed that miR-1277 takes part in biological process and several diseases. Cao et al showed that miR-1277 was significantly downregulated in HCC patients and HepG2 cells. Downregulation of miR-1277 promoted HepG2 cell proliferation, migration, and invasion [30]. Sugimachi et al also revealed that miR-1277 was downregulated in the bone marrow of patients with hepatocellular carcinoma [31]. However, the effects of miR-1277 on trophoblast cell proliferation, migration, and EMT have not been addressed. In our study, we demonstrated that miR-1277 silence accelerated trophoblast cell proliferation, invasion and migration, as well as EMT, while treatment with shRNA-CRNDE reversed the effects. These data suggested that CRNDE modulates biological function of trophoblast at least partly by regulating miR-1277.

Taken together, we disclosed that CRNDE expression was downregulated in PE tissues. The mechanistic results revealed that CRNDE had the accelerated effects on trophoblast proliferation, metastasis and the EMT formation by inhibiting miR-1277 expression, which provides a novel therapeutic target for clinical diagnosis and therapy of PE.

Acknowledgements

The study was approved by the Ethics Committee of Maternal and Child Health Care Hospital of Shandong Province, and all the patients signed an informed consent prior to enrolment.

Disclosure of conflict of interest

None.

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