

MicroRNA-24 inhibits CDX1 expression in decidual tissues of recurrent spontaneous abortion mice to reduce the abortion risk

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Abstract

Background. Recurrent spontaneous abortion (RSA), presenting as one of the difficult clinical diseases, has a high incidence rate among women of reproductive age, with a rising trend in recent years.

Objectives. To confirm a target relationship between miR-24 and CDX1. This study aimed to explore miR-24 expression in decidual tissue under recurrent spontaneous abortion (RSA) and its mechanism of regulating downstream gene *CDX1*.

Material and methods. Female CBA/J mice were mated with male BALB/C mice to establish normal pregnancy models, and mated with male DBA/2 mice to establish RSA models. Recurrent spontaneous abortion model mice were randomized into 5 groups: a model group, a NC group, a miR-24 mimic group, a CDX1 vector group, and a miR-24 mimic+CDX1 vector group. Expressions of miR-24, CDX1, VEGF, cleaved caspase-3, Fas, and FasL, as well as apoptosis in decidual tissues, embryonic development and embryo loss rate were compared.

Results. Compared with the normal group, the embryo loss rate, apoptosis rate, and the expressions of cleaved caspase-3, Fas and CDX1 in decidual tissue in other groups were significantly increased, and the expressions of miR-24, VEGF, and FasL were significantly decreased (all $p < 0.05$). The miR-24 mimic group showed the opposite changes when compared with the model group (all $p < 0.05$). However, CDX1 overexpression can significantly block the protective effect of miR-24 overexpression on embryonic development ($p < 0.05$).

Conclusions. MiR-24 can inhibit CDX1 expression in decidual tissue of RSA mice, thus improving the embryonic development of the mice and reducing the RSA risk.

Key words: spontaneous abortion, caudal-type homeobox protein 1, microRNA-24

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Introduction

Recurrent spontaneous abortion (RSA) is defined as 3 consecutive pregnancy losses in a woman of reproductive age in the setting of 1 identical sexual partner.^{1,2} Recurrent spontaneous abortion, presenting as a difficult clinical disease, has a high incidence rate among women of reproductive age, with a rising trend in recent years.^{3,4} Exploration on the mechanism of RSA can enrich the theoretical basis in clinical treatment and thus may provide new critical ideas for RSA treatment.

We found that caudal-type homeobox 1 (CDX1) was significantly overexpressed in RSA through preliminary experiments. No research at present has confirmed the mechanism of CDX1 affecting RSA. One research study showed that the expression of enhancer of zeste homologue 2 (EZH2) was significantly downregulated in RSA, and 1 other research study through chromatin immunoprecipitation assay confirmed that EZH2 was obviously bound with CDX1, and through dual-luciferase reporter gene assay verified that EZH2 could negatively regulate CDX1 expression.^{5,6} Therefore, we speculated that CDX1 may be a crucial target in RSA. However, the deeper significance and potential mechanism of CDX1 in RSA are still unclear. To date, many studies have reported microRNA expression regulation in RSA.^{7–11} Some studies have shown that miR-24 expression is downregulated in decidual NK cells.^{12–14} MiR-24 regulates post-transcriptional expressions mainly by targeting downstream genes. Collectively, miR-24 may also serve as an important regulatory factor affecting RSA. As a result, we screened the downstream target genes of miR-24 and found that there was a binding site between CDX1 and miR-24. Therefore, we further speculated that miR-24 may highly possibly serve as the upstream regulatory component of CDX1 in RSA.

In this study, we constructed RSA mouse models and conducted overexpression on miR-24 and CDX1 in order to explore the expression regulation relationship between miR-24 and CDX1 in the decidual tissues of RSA model as well as its effect on RSA.

Methods

Animals

One hundred and twenty specific pathogen-free female CBA/J mice, 10 male BALB/C mice and 50 male DBA/2 mice at 6–8 weeks of age were selected in this study. The mice were raised in animal rooms at suitable temperature, under a 12/12-hour dark/light cycle with ad libitum access to standard rodent diet and water throughout the study. All procedures were approved by the Animal Care and Use Committee and conformed to the revised (1996) Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH). This study was reviewed and approved by the local ethics committee.

Animal modeling and grouping

The normal pregnancy models were constructed by mating CBA/J female mice with BALB/C male ones, while RSA models were made by mating CBA/J female mice with DBA/2 male ones. Twenty CBA/J female mice and 10 BALB/C male ones were mated in a ratio of 2:1, while 100 CBA/J female mice and 50 DBA/2 male ones were mated in the same ratio.^{15,16} The presence of a vaginal plug marked that day as embryonic day 0 in a pregnant mouse, and finally 20 normal pregnancy model mice and 100 RSA model mice were obtained.

The RSA model mice were randomized into 5 groups on average, the model group (RSA mice without other treatment), the NC group (RSA mice with injection of miR-24 NC and transfection reagent through caudal vein), miR-24 mimic group (RSA mice with infection of miR-24 mimic and transfection reagent through caudal vein), the CDX1 vector group (RSA mice with injection of pcDNA3.1-CDX1 overexpression vector), and the miR-24 mimic+CDX1 vector group (RSA mice with injection of miR-24 mimic and pcDNA3.1-CDX1 overexpression vector). MiR-24 mimic NC, miR-24 mimic and pcDNA3.1-CDX1 were all purchased from Guangzhou RiboBio Co., Ltd., Guangzhou, China. MiR-24 mimic NC, NC vector, miR-24 mimic, and pcDNA3.1-CDX1 were mixed with 50 μ L culture medium and with 50 μ L EntransterTM-in vivo transfection reagent. According to the grouping, the mixed solutions were injected into the corresponding mice through the caudal vein at a dose of 2 mg/kg. The first intravenous injection was carried out at the time of mating in the cage; then, the same injection was performed at three-day intervals until the 14th day after conception. The mice were sacrificed at day 14 after conception, and the uteri of the mice were then removed out for observation on the embryonic development and the calculation on the embryo loss rate.

Quantitative reverse-transcriptase PCR

Decidual tissues of the mice in each group were taken and ground into homogenate for quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) analysis. Total RNA in the decidual tissues was extracted using TRIzol[®] reagent (Invitrogen, Carlsbad, USA). The extracted RNA was reversely transcribed into cDNA by the TaqMan[®] MicroRNA Reverse Transcription Kit (4427975; Applied Biosystems, Foster City, USA). The reverse transcription reaction volume was set as 25 μ L. The reaction was performed at 37°C for 30 min, and the inactivation reaction was done at 85°C for 5 s. Primers designed for miR-24, CDX1, GAPDH, and U6 were synthesized by Beijing Tsingke Biotechnology Co., Ltd., Beijing, China (Table 1). Reverse-transcriptase quantitative PCR was processed using ABI7500 real-time quantitative PCR instrument (Thermo Fisher Scientific, Waltham, USA). The reaction conditions were as follows: pre-denaturation at 95°C for 10 min, denaturation at 95°C for 10 s, annealing at 60°C for 20 s, and lastly extension at 72°C for 2 min,

Table 1. Primer sequences

Gene	Primer sequence (5'-3')
miR-24	F: GGGTGGCTCAGTTCAGCAG
	R: GTGCAGGGTCCGAGGT
U6	F: CTCGCTTCGGCAGCACATA
	R: AACGATTCACGAATTTGCGT
CDX1	F: GATGGCCTCAATGGGTACAAG
	R: TCGTTGTTGTTGATGTCACAGT
GAPDH	F: TGGCCTCCGTGTTCTCTAC
	R: GAGTTGCTGTTGAAGTCGA

F – forward; R – reverse; CDX1 – caudal type homeobox 1.

with 35 cycles. The PCR system was set as 20 μ L, containing 0.8 μ L of qPCR forward primer (10 μ M), 0.8 μ L of qPCR reverse primer (10 μ M), 0.4 μ L of ROX reference dye II, 10 μ L of SYBR® Premix Ex Taq™ II (Thermo Fisher Scientific), 2.0 μ L of cDNA template, and 6.0 μ L of sterilized distilled water. The U6 and GAPDH were used as internal controls for the relative expression of miR-24 and CDX1 mRNA, respectively. The relative expression level of each target gene was calculated with $2^{-\Delta\Delta C_t}$ method, where $\Delta\Delta C_t = \Delta C_{t_{\text{experimental group}}} - \Delta C_{t_{\text{GAPDH}}}$, in which $\Delta C_t = C_{t_{\text{target gene}}} - C_{t_{\text{internal control}}}$. C_t referred to the amplification cycles.

Dual-luciferase reporter gene assay

The binding sites of miR-24 to CDX1 gene within the 3'-UTR were analyzed through the bioinformatics prediction website (<https://cm.jefferson.edu/rna22/>). The target relationship between miR-24 and CDX1 was verified using dual-luciferase reporter gene assay. The 3' UTR of CDX1 gene and the 3' UTR of mutated CDX1 gene were respectively inserted into the dual-luciferase reporter gene vector pGL3-Basic, and 2 vectors were named as PGL3-CDX1 wt and PGL3-CDX1 mut, respectively. Renilla plasmid and the 2 reporter gene vectors were respectively co-transfected with miR-24 mimic and miR-24 mimic NC into human embryonic kidney (HEK) 293T cells. After 24 h of cell transfection, dual-luciferase activity was detected using the dual-luciferase reporter gene assay. The cells of each group were lysed and then centrifuged at 12,000 rpm for 1 min, and the supernatant was collected. The dual-luciferase reporter assay kit was purchased from Promega (Boston, USA) and luciferase activity was measured according to the instructions of the kit.

Western blotting

Western blotting was used to detect the expression levels of cleaved caspase-3, Fas, FasL, and VEGF in decidual tissues of the mice. Decidual tissue homogenate was again prepared for western blotting. Protein extraction was performed using RIPA cell lysis reagent (BB-3209; BestBio Technology, Co., Ltd., Shanghai, China). The proteins were

separated with SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane at a constant voltage of 80 V. After being sealed by blocking solution for 1 h, the membrane was incubated with anti-rabbit primary antibodies against cleaved caspase-3 (1: 500; ab49822; Abcam, Cambridge, UK), Fas (1: 1,000; ab15285; Abcam), FasL (1: 1,000; ab15285; Abcam), VEGF (1: 1,000; Ab27278; Abcam) and GAPDH (1: 2,500; ab9485; Abcam), and sealed for incubation with shaking overnight at 4°C. Then, the membrane was incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (1: 2,000; ab205718; Abcam) with shaking at 37°C for 2 h. The PVDF membrane was color-developed and photographed. The relative expression of a target protein = the gray value of the target band/the gray value of the internal control band.

TUNEL fluorescence assay

TUNEL fluorescence detection kit (Roche, Basel, Switzerland) was used to detect the cell apoptosis of decidual tissues in all mice. Paraffin blocks of fixed decidual tissues of all mice were made and sliced into serial sections. Then, the sections were mounted onto glass slides and numbered in sequence. Subsequently, the slices with sections were dewaxed and hydrated, and reacted with cell permeabilization reagent for 8 min, followed by washing with phosphate-buffered saline (PBS) twice. The 50 μ L TUNEL reaction mixture was added to the slides for 1 h in a wet box at room temperature, followed by 3 washes with PBS. The treated slides were observed under a Nikon eclipse TE2000-U fluorescence microscope (Nikon, Tokyo, Japan). The average number of TUNEL positive cells was observed using Image-Pro Plus v. 6.0 software by randomly selecting 5 visual fields under a light microscope.

Immunofluorescent assay

The decidual tissue sections of all the mice were made and then mounted onto glass slides. Slices were sealed with primary antibody mouse anti-VEGF (1: 100; ab205; Abcam), incubated at room temperature for 1 h and sealed by FITC conjugated goat anti-mouse IgG antibody (1: 200; ab150117; Abcam) for 30 min. Then, the nucleus was stained by Invitrogen™ Molecular Probes™ DAPI (4',6-diamidino-2-phenylindole, dihydrochloride) for 10 min. After ten-minute staining, the slides were washed 3 times with 1 × PBS for 10 min each time to prevent fluorescence quenching agent from entering the sealed sections. The slides were observed under a fluorescence microscope (XSP-BM22AY; Shanghai Optical Instrument Factory, Shanghai, China).

Statistical analysis

Data analyses were performed with the SPSS v. 21.0 software package (IBM Corp., Armonk, USA). All measurement data were expressed as mean \pm standard deviation

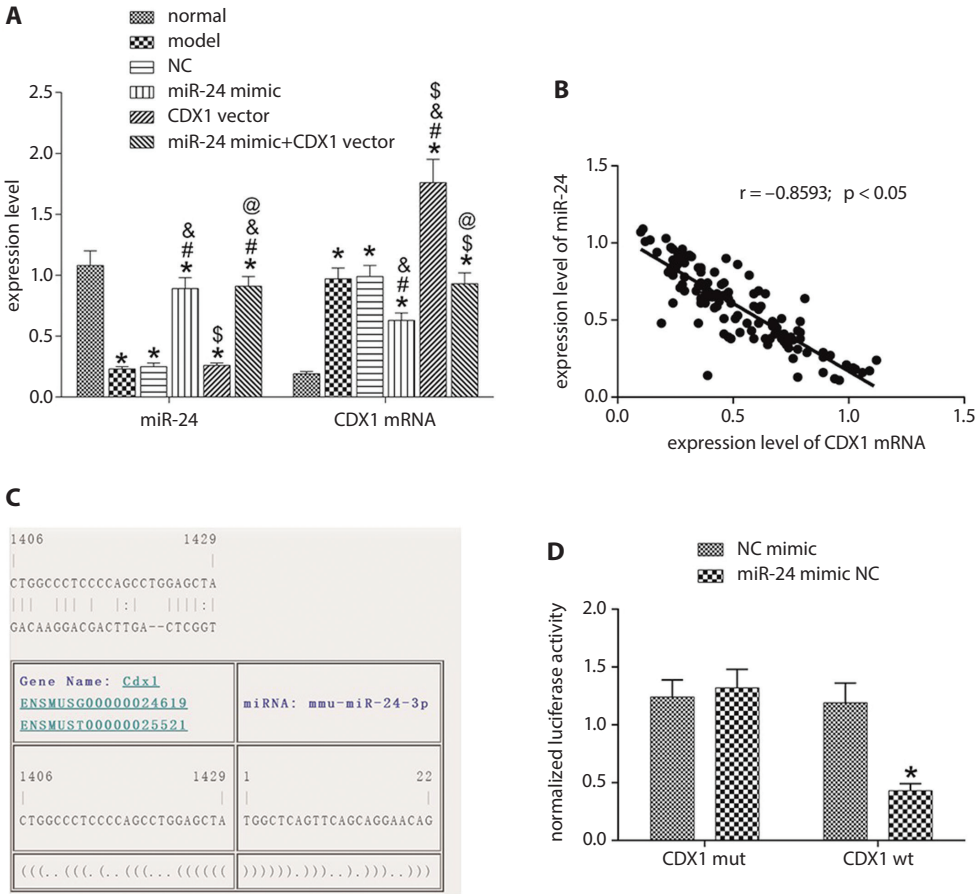


Fig. 1. MiR-24 can negatively regulate CDX1 expression. **A.** The expressions of miR-24 and CDX1 in decidual tissues of the mice through qRT-PCR; compared with the normal group, * $p < 0.05$; compared with the model group, # $p < 0.05$; compared with the NC group, @ $p < 0.05$; compared with the miR-24 mimic group, $^{\circ}p < 0.05$; compared with CDX1 vector group, $^{\circ}p < 0.05$; **B.** Correlation analysis between miR-24 and CDX1; **C.** The targeted relationship between miR-24 and CDX1 through dual-luciferase reporter gene assay; **D.** The results of dual-luciferase reporter gene assay. Compared with NC mimic, * $p < 0.05$

CDX1 – caudal type homeobox 1.

(SD). Pairwise comparisons among groups were conducted with one-way analysis of variance (ANOVA) test combined with Bonferroni post hoc test. A value of $p < 0.05$ was considered statistically significant.

Results

MiR-24 can negatively regulate CDX1 expression

The expressions of miR-24 and CDX1 in decidual tissues of the mice detected using qRT-PCR are shown in Fig. 1A. Compared with the normal group, miR-24 expression level significantly decreased but CDX1 significantly increased in other groups (all $p < 0.05$). Compared with the model group, miR-24 expression level in miR-24 mimic group was significantly increased, with significant decrease in CDX1 expression; miR-24 expression level in the miR-24 mimic+CDX1 vector group was significantly increased ($p < 0.05$), but with no significant change in CDX1 expression level ($p > 0.05$); CDX1 expression level in the CDX1 vector group was significantly increased ($p < 0.05$), but with no significant change in miR-24 expression level ($p > 0.05$). We found a significant negative correlation between miR-24 and CDX1 in 120 female mice as shown in Fig. 1B. Since a binding site between miR-24 and CDX1 was predicted

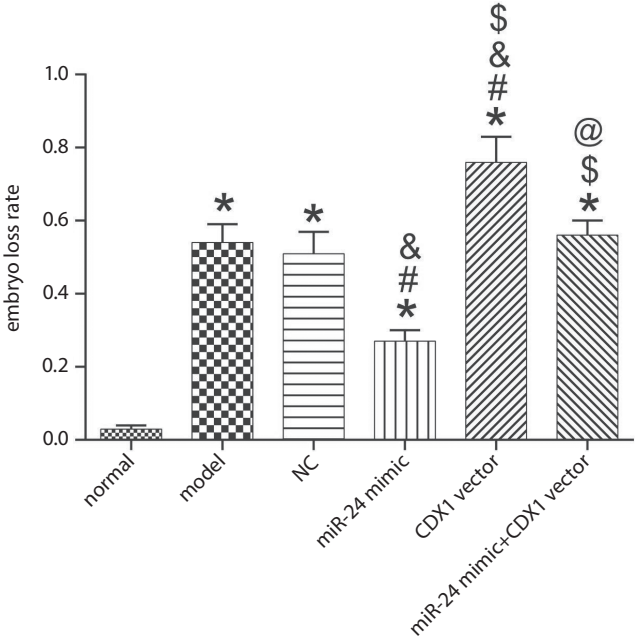


Fig. 2. Comparisons between groups of mice in embryonic loss rate. Compared with the normal group, * $p < 0.05$; compared with the model group, # $p < 0.05$; compared with the NC group, @ $p < 0.05$; compared with the miR-24 mimic group, $^{\circ}p < 0.05$; compared with the CDX1 vector group, $^{\circ}p < 0.05$

CDX1 – caudal type homeobox 1.

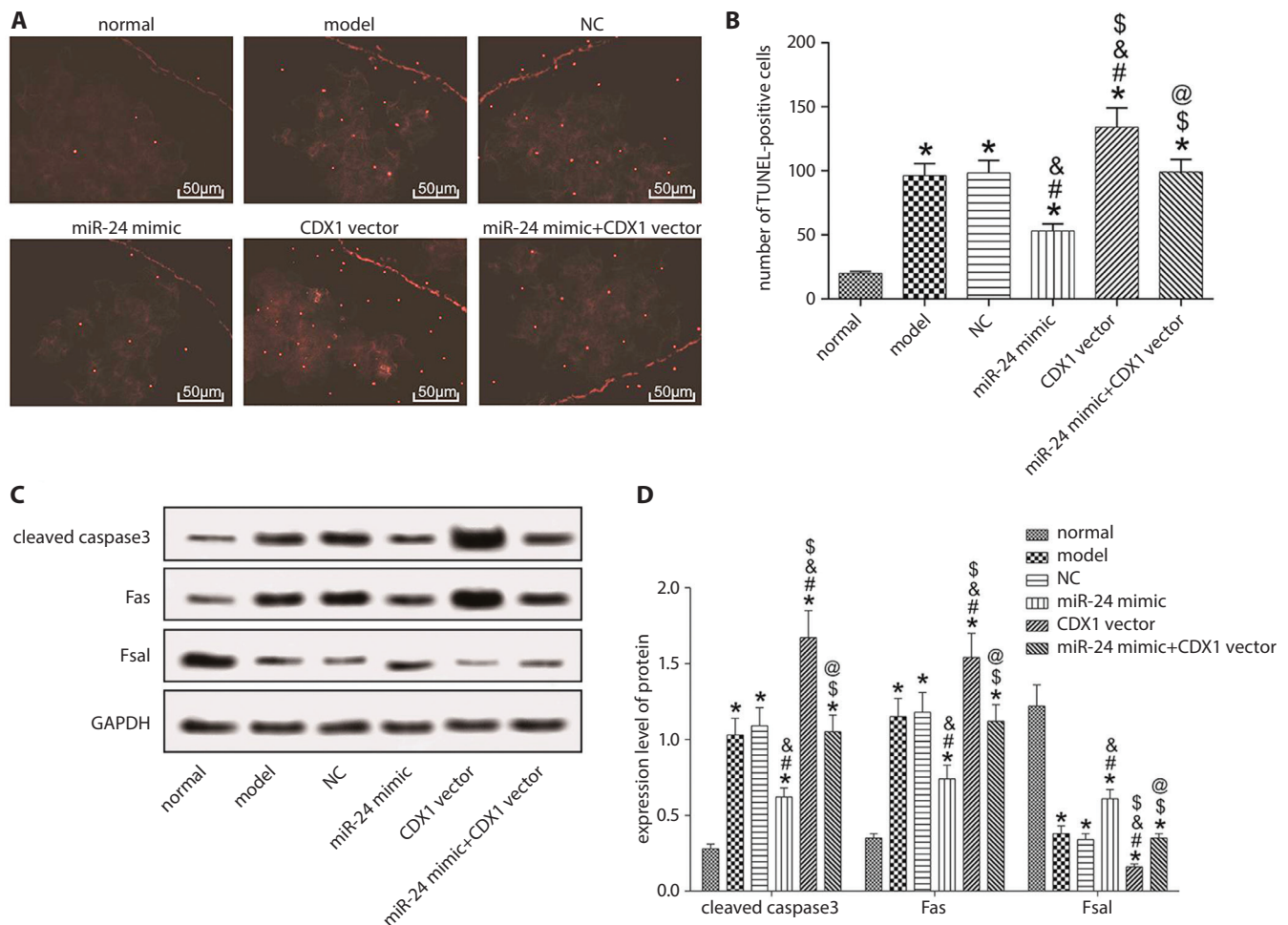


Fig. 3. Analysis of apoptosis in decidual tissues of the mice. The apoptosis (A) and the numbers of apoptotic cells (B) in decidual tissue of the mice through TUNEL fluorescence assay; C. Western blot bands; D. Detection results of apoptosis-related factors. Compared with the normal group, * $p < 0.05$; compared with the model group, # $p < 0.05$; compared with the NC group, \$ $p < 0.05$; compared with the miR-24 mimic group, @ $p < 0.05$; compared with the CDX1 vector group, & $p < 0.05$

CDX1 – caudal type homeobox 1.

by RNA22, we hypothesized that miR-24 may act as an upstream regulatory element regulating CDX1 expression, so we performed gene assay using a dual-luciferase reporter to test binding sites. The results in Fig. 1C and 1D showed that luciferase activity was significantly decreased after co-transformation of wild-type CDX1 reporter plasmid and miR-24 mimic ($p < 0.05$), indicating that miR-24 can targetedly and negatively regulate CDX1 expression.

Embryonic development of the mice

The mice were sacrificed at day 14 after pregnancy, and the uteri were then removed for observation for the embryonic development of all mice to obtain the embryo loss rate (Fig. 2). A higher embryo loss rates was observed in other groups when compared to that in the normal group (all $p < 0.05$). Additionally, when compared with the model group, opposite results occurred in the miR-24 mimic group, while similar results were obtained in the CDX1 vector group (both $p < 0.05$).

Cell apoptosis of decidual tissues of the mice

Cell apoptosis of decidual tissues may be one of the causes contributing to mouse embryo death and RSA. We detected the cell apoptosis of decidual tissues in all mice using TUNEL fluorescence assay, and the results are shown in Fig. 3A and 3B. The normal group has significantly more TUNEL positive cells than other groups. The number of TUNEL positive cells significantly decreased in miR-24 mimic group, but significantly increased in CDX1 vector group when compared to that in the model group, which was significantly elevated in the miR-24 mimic+CDX1 vector group when compared with that in the miR-24 mimic group, while significantly decreased in the miR-24 mimic+CDX1 vector group when compared to that in the CDX1 vector group ($p < 0.05$). Additionally, we detected the expression levels of apoptosis-related factors, cleaved caspase-3, Fas and Fsl, through western blotting. The results in Fig. 3C and 3D show that

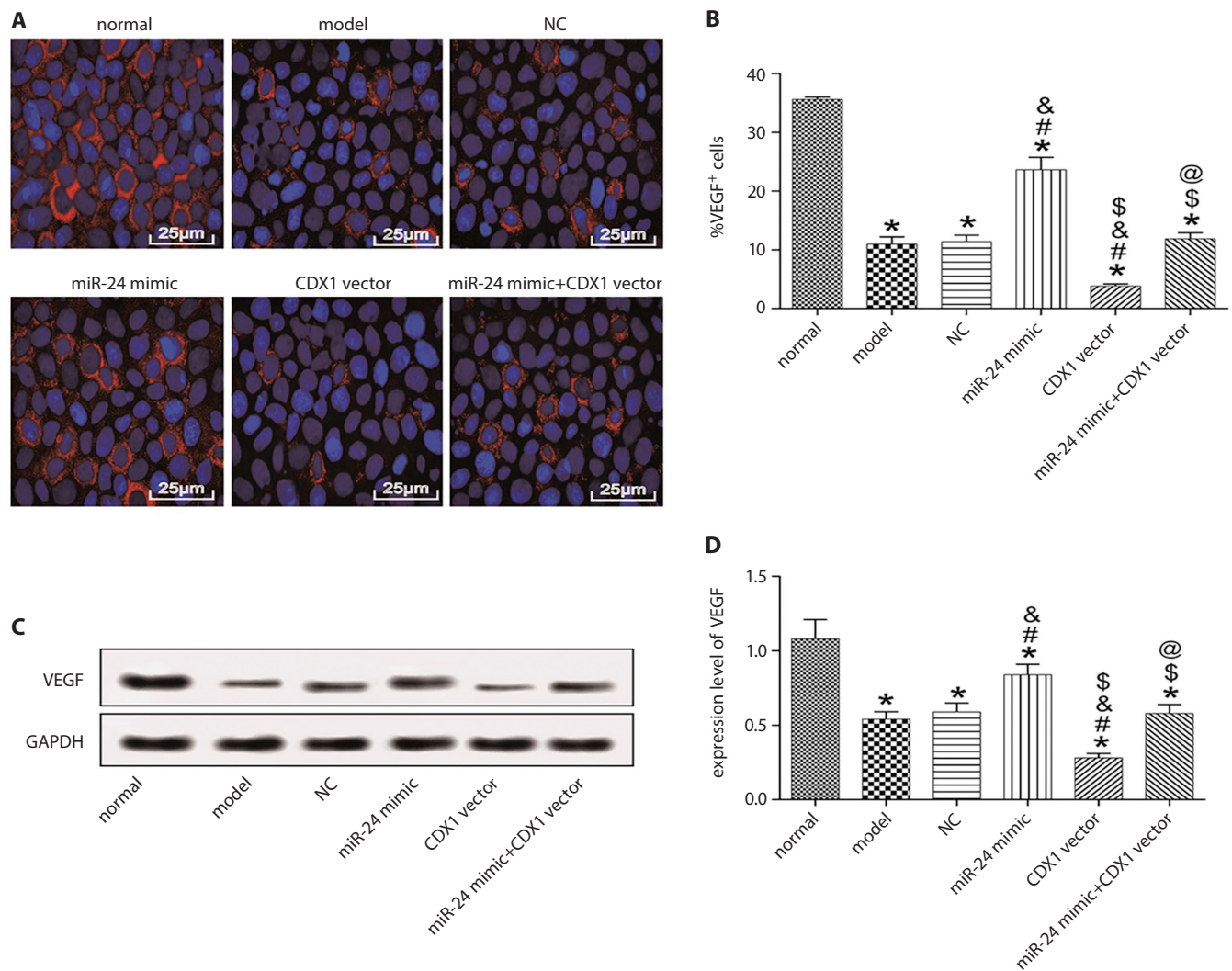


Fig. 4. Immunohistochemical results of rats in all groups. A. Microscopic image of immunohistochemical staining (×400 magnification). B. Statistical analysis of the number of VEGF-positive cells in all groups; C. Western blot bands; D. Statistical analysis of VEGF protein expression. Compared with the normal group, * $p < 0.05$; compared with the model group, # $p < 0.05$; compared with the NC group, $^{\circ}p < 0.05$; compared with the miR-24 mimic group, $^{\$}p < 0.05$; compared with the CDX1 vector group, $^{\circ}p < 0.05$

CDX1 – caudal type homeobox 1; GAPDH – glyceraldehyde-3-phosphate dehydrogenase.

overexpression of miR-24 could inhibit the expressions of cleaved caspase-3 and Fas, and promote the expression of FasL, while CDX1 overexpression showed the opposite changes. These results comprehensively confirmed that miR-24 overexpression can significantly reduce the cell apoptosis of decidual tissue, while overexpression of CDX1 can promote the apoptosis of decidual tissues in mice; moreover, CDX1 overexpression can block the inhibitory effect of miR-24 overexpression on the apoptosis of decidual tissues in mice.

VEGF expression of decidual tissues of the mice

The expression of VEGF in decidual tissues was detected with immunofluorescence assay, and the results are shown in Fig. 4A and 4B. The normal group has significantly higher fluorescence expression levels of VEGF in decidual

tissues than other groups ($p < 0.05$). The VEGF fluorescence expression was significantly higher in miR-24 mimic group, while significantly lower in CDX1 vector group when compared to that in the model group ($p < 0.05$). Additionally, we detected the protein expression of VEGF using western blotting (Fig. 4C,D), and the results were consistent with expression trend in the immunofluorescence assay.

Discussion

Recurrent spontaneous abortion presents high incidence among pregnant women, with high difficulty in treatment and cure. The mechanism of RSA under exploration may serve as a crucial guiding role in clinical treatment.^{17,18} In this study, we mainly focused on decidual tissue, which plays a vital role in the occurrence and maintenance of a pregnancy.¹⁹

Initially, we found that CDX1 may be overexpressed in RSA through preliminary experiments. Meanwhile, some literatures showed that CDX1 may have aggravating effect on the condition of RSA. Therefore, we constructed RSA mouse models and detected the expression of CDX1 in normal pregnant mice and RSA mice, and the results were consistent with the results of the bioinformatics prediction. Thus, we performed overexpression on CDX1 in RSA mice. The results showed that the fatality rate of treated mouse embryos increased significantly, suggesting that the development of mouse embryos evidently deteriorated after overexpression of CDX1. Additionally, we detected the apoptosis of decidual tissues and found that the apoptosis rate of decidual tissues in the mice increased significantly after CDX1 overexpression. VEGF plays a highly favorable role in the auxoaction for vascular remodeling of endometrium tissue, thus further facilitating differentiation of trophoblastic cells of mouse embryo and embryo implantation, conclusively promoting mouse embryo development.^{20–22} We found that the expression level of VEGF after overexpression of CDX1 decreased significantly. Based on the above experiments, we speculate that CDX1 overexpression in the RSA mice may not only lead to apoptosis of decidual tissue and instability of embryo development during pregnancy, but also cause inhibition on VEGF expression and embryo implantation, thus hindering the development of mouse embryo and giving rise to further occurrence of RSA.

The bioinformatics prediction website also found a binding site between miR-24 and CDX1. We also detected the expression of miR-24 in normal pregnant mice and RSA mice, and verified the binding relationship between miR-24 and CDX1 using dual-luciferase reporter gene assay. The results show that miR-24 expression was down-regulated in RSA mice, and miR-24 could target CDX1 and inhibit its expression. The phenotypic results in RSA mice receiving overexpression on miR-24 were opposite to those receiving overexpression on CDX1. In addition, compared with the RSA mice receiving miR-24 overexpression, the apoptosis ratio of decidual tissue in the RSA mice treated with overexpression on both miR-24 and CDX1 was significantly elevated, with significant reduction on VEGF expression. Based on the above results, we speculate that miR-24 overexpression can reduce apoptosis of decidual tissue and increase VEGF expression by inhibiting CDX1 expression in RSA mice, thus improving mouse embryo development and lowering abortion rate.


However, the causes of RSA are complex, and contributors also include endocrine disorders, body immunity and prethrombotic state apart from the effects of decidual tissue and uterus implantation.^{23–28} However, this study mainly aimed to explore the effects of miR-24 and CDX1 on apoptosis of decidual tissue and VEGF expression, and it is not clear whether miR-24 and CDX1 have regulatory effects on endocrine, immune function and prethrombotic state of pregnant mice.


Conclusions

We confirmed the expressions of miR-24 and CDX1 in decidual tissues of RSA mice and also the expression regulation relationship between them, which further enriches the mechanism of RSA, potentially providing a new idea for clinical treatment on RSA.

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